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TUMORAL AMYLOIDOSIS OF THE LUNG

By

BERTIL FORS and LARS RYDÉN

Received 10.x 63

Authors who have recently published cases of atypical, primary, localized amyloidosis of the lung, commonly called tumoral amyloidosis, have expressed the wish that all new cases encountered be reported, to provide a better basis for the assessment and elucidation of the mode of genesis of the change A case is now presented

CASE REPORTS

The case report concerns a 95 year old woman who had been healthy until the last 3 years of her life when she started to show increasing mental disorientation loss of weight and weakness She was not bedridden however One week before death she had to be removed to a home for the aged owing to her mental state On admission she was fairly bright Examination disclosed signs of disorientation in time and space There was

Clinical diagnosis —Cerebral and cardiac arteriosclerosis with hypertonia Sideropenic anaemia (Cause of death unknown)

Necropsy The body was that of an elderly woman with wasting of tissues and muscular atrophy

The brain weighed only 1000 g The sulci were greatly

The upper respiratory passages were filled

The cut surface of

The liver weighed 1 180 g and showed stasis

The kidneys weighed 165 g together The cortex was thin with a few retention cysts the size of peas

The

Ther

Hist

layer

occasional



Gieson's stain Shadows of the alveolar network were detectable here and there ... sclerotic band were seen in the ... ches were poorly ... ther wide vessels ... rly large numbers ... le capillary walls ... mphocytes and a ... na cells were very ... vessels took ... staining re- ... tions stained ... (20) aniline ... en had been fixed in formalin Ladewig stained sections showed small red areas in the capsule On the basis of these findings the homogeneous material was taken to consist of amyloid In ... in the vesse ... ma like (Fi ... fungi could ... No amylo ... material and slight granulocyte infiltration were present in many bronchi both coarse and fine

...nodular amyloidosis of the left lower lobe of the lung (secondary to ?haem angioma? hamartoma) Hepatic stasis
Fibroleiomyoma of the uterus
Cause of death aspiration bronchitis

DISCUSSION

Many authors have put forward classifications of amyloidosis *e g* (*King* (20), *Lanzenauer* (22), and *Reiman et al.* (25)) *Weiss* (32) has given a schema with regard solely to pulmonary amyloidosis

I Secondary amyloidosis
II Senescent cardiac amyloidosis
III Amyloidosis associated with myelomatosis
IV Primary amyloidosis
  A Generalized primary amyloidosis
    1 Nodular pulmonary involvement
    2 Diffuse pulmonary involvement
  B Amyloidosis limited to respiratory tract
    1 Upper respiratory tract and lungs
    2 Isolated nodular pulmonary amyloidosis

Figs 1 2

Fig 1 Section through the lower lobe of the left lung showing a tumour in the upper part

Fig 2 Peripheral portion of the tumour showing islets of bone and cartilage (× 95 van Gieson's stain)

2



1

A few cases of focal pulmonary amyloidosis with 'typical' secondary amyloidosis have been encountered in the literature *Lunzenauer* (22) has published a case of multiple, focal amyloidosis and the lung with deposits of amyloid in other organs, in association with tuberculosis (Weiss Group I) Atypical and probably secondary to multiple myelomatosis (Group III) is the focal amyloidosis of the case described by *Weiss* (32) The amyloidosis in *Duke's* (7) case (Group IV A 1) would seem to be generalized, primary, and atypical, and occurring in other organs including the liver, spleen, lymph nodes, kidneys and testis Similar cases have been described by *Ferris* (9) and *Holle* (17) *Glauser* (11) and *Herxheimer* (16) have published cases of atypical, primary amyloidosis which in the lung was focal but in which there were amyloid deposits in the larynx, trachea, and bronchi (Group IV B 1) Cases of localized, atypical, primary amyloidosis of the lung (Group IV B 2), to which our case may be referred, are few and far between in the literature This form of the disease has often been called 'tumoral or tumour-forming amyloidosis' As far as we have been able to determine, altogether 17 cases have been reported in which the changes, solitary or multiple, have been strictly limited to the lung tissue These are given, together with our case in Table 1

Cases of amyloidosis limited to the larynx, trachea, and bronchi appear to be very much commoner, and we have seen at least two Such cases have been described by several authors, including *Balser* (1), *Bauer* (2), *Glockner* (12), *Schottenfeld et al* (28), *Weisman et al* (31), *von Werdt* (33), and *Ogawa* (24)

The histological investigation in our case has shown certain interesting features, namely the extensive patches of dense, angioma-like structures These suggest that the amyloid lesion has developed into a vascular tumour or granuloma

[illegible] n, and m [illegible] event the [illegible] essels, cy [illegible] secondary polycythaemia No such changes were present in our case, but there was marked sideropenic anaemia Capillary angiomata are usually small, increase in size only slowly, and are commonly multiple or present concomitantly with cavernous angiomata It is conceivable that a slowly developing lesion of such nature preceded the localized amyloidosis in our case

Thrombosis or embolus may, when organized, result in an angioma like change, known as 'angiomatoid lesion' (*Gould* (13)) It is possible

---

Figs 3–4

Fig 3 The homogeneous amyloid parts of the tumour with scanty cells (× 100 van Gieson's stain)

Fig 4 Part of the tumour showing structures reminiscent of the alveolar network (× 180 Haematoxylin eosin)

that a lesion of this type was present in our case The age distribution of patients with amyloidosis of Group IV B 2 and the heart disease common among them would well tally with such a relationship (see Table 1) In our case there was no source of emboli and no signs of pulmonary hypotension Schistosomiasis a disease process that *Shaw & Ghareeb* (27) found to produce such changes is out of the question in this patient And no other parasite or micro organism has been demonstrable

Islets of cartilage and or ossification have been demonstrated in practically all cases of amyloid tumour mostly in the peripheral parts (*Bergman & Linder* (4) *D Arrigo* (6) *Gery* (10) *Hallerman* (14) *Hommerich* (18) *Lunenauer* (22) *Meyer* (23) *Weiss* (32) Ossification is a rather unspecific process that could well take place in an angioma with haemorrhage an old infarct and old inflammatory focus or a hamartoma The islets of cartilage have been regarded as represting persistent bronchial cartilage (*Bergman & Linder* (4) and others) but in our case at any rate the structure of the cartilage is distorted in places showing a certain tendency to proliferate The new formation of bone and cartilage would accord better with a structure of the hamartoma type than with an infarct or inflammatory focus in the lung Another possibility is that the bony and cartilaginous proliferation is a phenomenon secondary to the amyloidosis possibly facilitated by the presence of the acid mucopolysaccharides that constitute part of the amyloid substance This last named theory receives some support from *Lessers* (21) case in which the bony tissue after decalcification showed positive staining reactions for amyloid

A feature that pulmonary angioma and pulmonary hamartoma have in common is that they may run a silent course and be discovered only by chance for example on mass radiographs or necropsy The same is true of the cases of amyloid tumour that have been reported (see Table 1) In cases in which there have been symptoms these have largely been pressure symptoms such as cough and occasionally haemoptysis (*Bergman & Linder* (4) *Haynes et al* (15) *Weiss* (32) *Weinberg* (34))

It is possible that degeneration and breaking down of t[illegible]

[illegible] and stroma of malignant tumours In certain cases the substances shows metachromasia (Fig 7) Tumours that show regression for example hypernephroma (*Hultquist* (19)) and others may therefore conceivably become transformed into amyloid

Figs 5–6

Fig 5 The haemangioma like parts of the tumour showing amyloid deposits in the vessel walls (× 160 Methyl violet)

Fig 6 The angioma like parts after silver impregnation (× 190 Laidlaw s stain)

b

1
... Strictly Locali ed to the Lung

Tumoral amyloidosis			Diagnosed at	Illness leading to death	Remarks
Site	Number	Size			
LLL	1	5 5 × 15 cm	Necropsy	Heart failure due to mitral insufficiency Mitral and aorta stenosis	
Bil	Mult	Bean hen s egg	Necropsy	Cerebral haemorrage	
Right lung	Mult	0 6 × 0 4 cm	Necropsy	Fracture of the femur	
Bil	Mult	Up to 2 cm	Necropsy	Bronchopneumonia	
RML RLL LLL	7	Pea 2 cm	Necropsy	Postoperative pneumonia Right sided empyema	
RLL	2	Pea	Necropsy	Pneumonia	
Bil	Mult	1 2 cm	Necropsy	Cardiac failure	
RML	1	6 cm	Pneumonectomy		
Bil	Mult	1 2 cm	Necropsy	Cardiac failure Serum protein 9 07 gm/100 ml	
Bil	Mult	9 cm	Necropsy	No data	
RLL	1	2 cm	Necropsy	Cerebral haemorrage	
Bil	Mult	Miliary	Necropsy	Laryngeal oedema Cardiac failure	
LLL LLL	4	6 cm and 3 cm	Thoracotomy		Leftsided costal facture 2 years earlier
?	?	?	Thoracoscopy		
Bil	Mult	0 5–4 cm	Necropsy	Cerebral abscess	(Myelomatous tumours of the lung)
Bil	Mult	?	Necropsy	?	
LLL LLL	15	4 × 5 cm	Pneumonectomy		End cervical carcinoma 7 years earlier
LLL	1	5 × 6 cm	Necropsy	Aspiration bronchitis	

middle and the lower lobes of the right lung Bil = bilateral, Mult = multiple,

TA
Cases of Tumoral Amyloi

Author	Age	Sex	Clinical signs	X-ray findings
1 Lesser, A (21)	78	F		
2 Meyer O (23)	57	M		
3 Ecoffey M (8)	88	M		
4 *Gery I* (10)	85	F		
5 Hellerman W (14)	52	F		
6 Hallerman W (14)	73	F		
7 D Arrigo S (6)	67	M		
8 Haynes A I et al	66	M	Cough dyspnoea (6 weeks)	Tumour of the right lung
9 Hommerisch K W (18)	70	M		
10 Lunzenauer K (22)	83	F		
11 Candiani G (5)	83	M		
12 Tennstedt A (30)	66	M		
13 Bergman L Linder B (4)	67	M	Cough respiratory infections (2 months)	3 tumour like processes in left lung
14 Winberg T (34)	72	F	Cough (5 6 months)	X ray signs (8 years)
15 Weiss L (32)	46	M	Cough (1 year)	Nodules (9 years)
16 Becker B J P (3)	82	M		
17 Schuller H (26)				
18 Present authors	95	F		

LUL LLL = the upper and lower lobes of the left lung RUL RLM and RLL = the upper,
F = female M = male

SUMMARY

A case of localized atypical primary amyloidosis of the lung is reported The tumour was encapsulated and in the peripheral parts bony and cartilaginous islets could be seen In some places there were also angiomatous structures The possibility of the existence of some precursory disease and its nature are discussed on the basis of the histological picture Seventeen similar cases already published are surveyed

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Fig 7
Poorly differentiated mammary carcinoma with vessels showing amyloidosis (× 95, Methyl violet)

tumours In the case of *Schuller et al* (26) the possibility of a precursory metastasis from a cured endocervical carcinoma is suggested

Stress has been laid in most earlier accounts on the presence of giant cells and plasma cells In our case too we noted a few large plasma cells, but they did not dominate the picture For this reason, and having regard to the only slightly raised sedimentation rate, it is highly unlikely that a plasmacytoma can have caused the localized amyloidosis

With this paper we have aimed to draw attention to certain different aetiological possibilities that should be borne in mind in the study of future cases of localized pulmonary amyloidosis The investigation of our case have aroused a strong suspicion that the deposition of amyloid was preceded by a tumour—probably an angioma or hamartoma

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THOROTRAST TUMOURS

Report of 3 Cases and a Microradiological Study of the Deposition of Thorotrast in Man

By

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Thorotrast was widely used as an x-ray contrast medium from the end of the 1920s to the beginning of the 1940s. It is colloidal solution of thorium dioxide. Thorium is radioactive with a half life period of 1.4×10^{10} years. The radiation emitted consists mainly of alpha rays. When injected into the vascular system only small amounts of thorotrast are excreted and most of it is deposited in the reticuloendothelial system. The largest quantities are found in the liver and the spleen.

During the last 20 years there have been reported several cases of tumours following the administration of thorotrast (cf. *Dahlgren* 1961). As pointed out by the previous authors it is desirable to report all cases of thorotrast tumours observed because thorotrast is one of the few radioactive materials with long half life that have been administered to human beings in comparatively large doses. A large material of individuals who have received thorotrast is therefore of interest in the study of the action of radioactive materials on the human body. Furthermore the first reported tumours had a short induction period (i.e. the time between thorotrast administration and the establishment of the diagnosis of a malignant tumour was brief) and may therefore have other characteristics than tumours that appear after long intervals after the injection of thorotrast. We found therefore it worth while to report another three cases which had long induction periods. One of these (Case 1) has previously been reported by *Larsson* (1961) from a somewhat different point of view.

METHODS

... with routine

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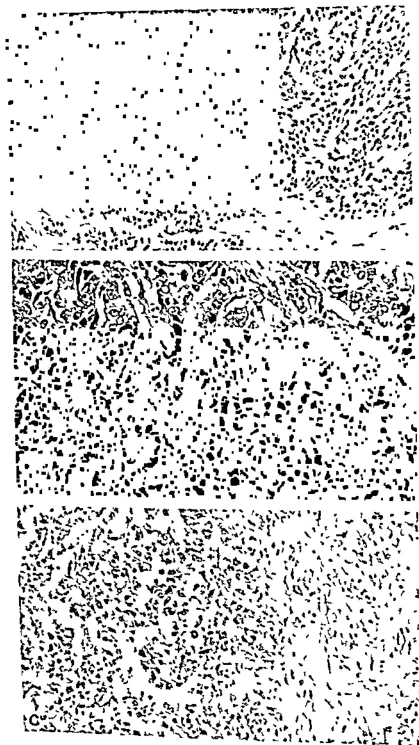


Fig 1

TABLE 1
Survey of Our Cases

Case	Tumor of the liver	Purpose of injection	Dose	Latency period (years)	Sex	Age at death
1	*Adenoacanthoma*	Cerebral angiography	17 ml	27	M	53
2	Adenocarcinoma	Cerebral *angiography*	?	21	M	74
3	Haemangioendothelioma	Cerebral angiography	30 ml	21	M	47

Microradiological examinations were performed on slices from the liver of Case 1 from the liver and the mediastinal tumour of Case 2 and from the liver, the spleen the *lymph nodes, the bone marrow of the femur diaphysis and the soft* tissues around the carotid arteries of Case 3 The slices varied in thickness between 1.5 cm and 500 μ. The thin sections were cut on a freeze microtome. The radiation was generated at 10–55 kV from a Philips x-ray diffraction tube (type no 25633/62) with an extremely fine focus (0 16 mm²) *The film-focus distance varied* between 40 and 110 cm The photographic material was Kodak Maximum Resolution and Gevaert Scientia 51.56 The slices were stored in thin-walled plastic-bags in order to prevent drying during the x-ray examination

CASE REPORTS

Case 1

Brief clinical history Male builder, born 1908 In 1933 carotid angiography with 17 ml thorotrast on account of a suspected brain tumour. Since *1960* increasing pain in the back and asthenia Plain film of the abdomen: radiopaque material in the spleen and around the 12th thoracal vertebra E S R 85 mm (Westergren method) Haemoglobin 58 per cent Alkaline phosphatases 46 units (King-Armstrong) Total *scintigram 198 Au. high uptake in the liver, femur, and around the knee joints;* no uptake in the spleen An exploratory laparatomy was performed with extirpation of the spleen, and a biopsy specimen was taken from the liver, which in one lobe showed superficial, scarred retractions of the surface The spleen was normal sized, it had a *firm consistency, the cut surface showed a pale yellow granularity* After operation rapid deterioration and death after two months

Histological examination of the specimens obtained at operation Dark granulous pigment in the periarteriolar connective tissue in the spleen and atrophy of the Malphigian corpuscles In the liver there was fibrosis and dark, granulous pigment in the interstitial connective tissue

Autopsy The body was that of a cachectic man with slight jaundice A large, necrotic tumour was found centrally in the right lobe of the liver *The capsule of the left lobe* was *thickened* and whitish with scarred retractions of the surface The liver weighed 1800 g The tumuor tissue was invading the left suprarenal gland, the 12th thoracal

---

Fig 1

Sections through the tumours of Cases 1 (A), 2 (B), and 3 (C) 130 × Haematoxylin eosin













Fig. 2

vertebra, the peritoneum, the lymph nodes and the periglandular tissue around the aorta The tumour masses compressed the aorta, the inferior caval vein, and the portal vein The inferior caval vein was filled with thrombotic material Several small emboli in the peripheral parts of the pulmonary arteries No oesophageal varices The femur contained red, active marrow No local changes around the carotid arteries on the neck Apart from a horse-shoe kidney, a small fibroma of the renal marrow, and a slight atrophy of the testis, there were no further relevant gross changes

Histological examination of autopsy specimens The tumour in the liver showed a rather typical appearance of a adenoacanthoma (cf Fig 1) It was built up of epithelial cells, the majority of which were clear and polygonal, mostly growing in compact strings and forming intercellular bridges in places, but showing no marked keratinization In some places there were small, dark cells growing in acinar formations Tumour tissue of the same appearance occurred in the pancreas, the left supra renal gland and the periaortal lymph nodes The remaining parts of the liver parenchyma showed fibrosis and granular accumulations, which stained brownish with van Gieson's stain and purple with hema toxylin-eosin Solitary granules of the same appearance were observed in the bone narrow and the lymph nodes There were no granules in the testis

Microradiological examination The x-ray pictures of the liver slices showed a pattern of radiopaque material that was similar to that of the liver in Case 3 (cf Fig 2) The amounts of radiopaque material were smaller than in the liver of Case 3, probably because the formalin had dissolved much of the material In histological sections through the slices that had been investigated by microradiography, it was easily seen that the radiopaque areas corresponded to granulous pigment which had stained brownish with van Gieson and purple with haematoxylin-eosin Largely identical pictures were obtained at 10 and 55 kV respectively

Diagnosis Thorotrast deposits in the spleen, the liver, the lymph nodes and the bone marrow Adenoacanthoma in the liver with metastases to the lymph nodes, the 12th thoracal vertebra, the peritoneum, and the left suprarenal gland Fibrosis of the liver and the spleen Thrombosis of the inferior caval vein Multiple small thrombemboli in the pulmonary arteries

---

Fig 2

Radiograms from the spleen (A C) and the liver (B D) of Case 3 A and B were from 7 mm thick slices Natural size C and D were 1 mm thick and enlarged 9 × Histological sections through the same spleen (F) and liver (I) stained with haemotoxylin eosin enlarged 12 × and photographed with a red filter to demonstrate the thorotrast granules (A and B were copied directly from the x ray film whereas the microradiograms C and D had to be photoed in a microscope Therefore the thorotrast is black in A and B and white in C and D)

Autopsy The body was that of a cachectic man with intense jaundice in the skin and the sclerae The abdominal cavity contained 600 ml brownish fluid The liver weighed 3240 g The capsule of the liver was thickened with whitish scarred retractions of the surface Centrally in the liver there was a grayish haemorrhagic tumour which had a diameter of about 9 cm The tumour showed large necroses It compressed the large bile ducts The spleen was somewhat enlarged with increased consistency the parenchyma contained miliary white densifications in the middle of the spleen there was a white fibrotic portion with a diameter of roughly 1 cm There were slight oesophageal varices Some paraaortic lymph nodes were enlarged and contained whitish haemorrhagic tumour tissue In both lungs a large number of whitish haemorrhagic tumour metastases were found In the central nervous system there was in the right temporal lobe a tumour metastasis which had a diameter of about 5 mm in the left occipital lobe there were the residues of the cerebral abscess No local changes around the carotid arteries Red active marrow in the femur diaphysis Apart from moderate general arteriosclerosis no pertinent changes were found grossly

Histological examination The tumours in the liver spleen lungs and brain showed a fairly typical appearance of a haemangiosarcoma with irregularly arranged cells about abundant blood spaces (cf Fig 1) the cytoplasm of the tumour cells was large and clear the nuclei were small and vesicular and a moderate number were undergoing mitosis Dark granules were found in large amounts in the liver and the spleen (cf Fig 2) Solitary granules were observed in the bone marrow and the paraaortic lymph nodes The granules were purple in sections stained with haemotoxylin eosin and brownish with van Gieson s stain The liver and the spleen showed fibrosis

Microradiological examination As seen from Fig 2 there were somewhat different patterns of distribution of the radiopaque material in the spleen and the liver In the liver much of this material was deposited in the periportal connective tissue and in clumps just beneath the capsule In the spleen there was a thin uniform capsular layer and a regular deposition of radiopaque material in the periarteriolar connective tissue of the parenchyma In the lymph nodes and in the bone marrow only solitary small granules of radiopaque material were observed There was no radiopaque material around the carotid arteries on the neck

Diagnosis Thorotrast deposits in the liver spleen paraaortic lymph nodes and bone marrow Haemangiosarcoma of the liver with metastases to the lungs the brain and the lymph nodes Fibrosis in the liver and the spleen

DISCUSSION

Dahlgren (1961) has recently reviewed and discussed the literature on thorotrast tumours *in extenso* We will therefore only discuss some remarkable data in own our cases

Case 2

Brief clinical history Farmer, born 1887 In 1941 probably thorotrast angiography in connection with a neurological investigation for right-sided hemiparesis In 1961 a tumour, as large as an orange, was found at the site of the thymus in connection with x ray examination of the lungs Plain film of the abdomen showed radiopaque material in the spleen and the liver In 1962 the patient died suddenly in the clinical picture of acute enterocolitis with bloody stools and shock

Autopsy The body was that of a cachectic man with intense jaundice in the skin and sclerae Centrally in the right lobe of the liver there was a whitish, cystic tumour, which had a diameter of about 7 cm In addition, the liver parenchyma contained several small white tumours The liver weighed 2450 g The right suprarenal gland had been invaded by the same tumour masses as the liver The intestines showed acute enterocolitis In the right kidney a tumour was found which had a diameter of roughly 3 mm At the site of the thymus, a tumour occurred which had a diameter of about 5 cm The tumour could be prepared free from the pericardium and the lungs It was encapsulated and had a whitish, partly necrotic, cut surface Advanced arteriosclerotic changes in the abdominal aorta with an aneurysm Cardioarteriosclerosis Partially occluding thrombosis of the coeliac and the superior mesenterial arteries Pulmonary oedema Congestion of the liver and spleen

Histological examination The tumour tissue of the liver showed a rather typical appearance of an adenocarcinoma which had a large irregular cells with pale polymorphous nuclei (see Fig 1) The carcinoma showed ingrowth into the vessels The central part of the tumour was necrotic The same carcinoma was found in the right *kidney and the right suprarenal gland In the remaining liver parenchyma* there were granules which with van Gieson's stain were brownish and with haematoxylin-eosin purple The mediastinal tumour showed the histological picture of thymoma of the "spindle-cell type", which was built up of spindle-shaped *cells surrounded by small, round* cells with scarce cytoplasm

Microradiological examination The picture was the same at that described for Case 1 There was no radiopaque material in the thymoma

Diagnosis Thorotrast deposits in the liver and the spleen Adenocarcinoma of the liver with metastases to the right kidney and suprarenal gland Fibrosis of the liver and the spleen Thymoma Severe generalized arteriosclerosis Arteriosclerotic aneurysm of the abdominal aorta Partially occluding thrombosis of the coeliac and superior mesenterial arteries Pulmonary oedema Enterocolitis

Case 3

1915 In 1941 left cerebral abscess
n arteriography with 30 ml thoro
ray examination revealed radio
1962 increasing abdominal pains

Autopsy The body was that of a cachectic man with intense jaundice in the skin and the sclerae The abdominal cavity contained 600 ml brownish fluid The liver weighed 3240 g The capsule of the liver was thickened with whitish, scarred retractions of the surface Centrally in the liver there was a grayish, haemorrhagic tumour which had a diameter of about 9 cm The tumour showed large necroses It compressed the large bile ducts The spleen was somewhat enlarged with increased consistency, the parenchyma contained miliary, white densifications, in the middle of the spleen there was a white, fibrotic portion with a diameter of roughly 1 cm There were slight oesophageal varices Some paraaortic lymph nodes were enlarged and contained whitish, haemorrhagic, tumour tissue In both lungs a large number of whitish, haemorrhagic tumour metastases were found In the central nervous system there was in the right temporal lobe a tumour metastasis which had a diameter of about 5 mm, in the left occipital lobe there were the residues of the cerebral abscess No local changes around the carotid arteries Red, active marrow in the femur diaphysis Apart from moderate general arteriosclerosis no pertinent changes were found grossly

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DISCUSSION

Dahlgren (1961) has recently reviewed and discussed the literature on thorotrast tumours *in extenso* We will therefore only discuss some remarkable data in own our cases

In all our cases, the thorotrast had probably been administered intraarterially and the primary tumour was in the liver, which seems to be the most common primary location for tumours after intraarterial injection of thorotrast (*Dahlgren* 1961) The induction period was longer in our cases than in most cases of thorotrast tumours previously reported Thus the mean induction period was 23 years in our cases and 17 7 years in the cases described in the literature up to 1962 (*Dahlgren* 1962) On the other hand, the dose of thorotrast injected was lower in at least two of our cases than in most cases previously reported *Bazerga* (1960) states that the induction period for liver sarcomas is 12 6 ± 7 4 years and considerably longer (19 4 ± 4 0 years) for carcinomas In our material, the mean induction period for our two cases of carcinoma was also longer (24 years) than that for the case of sarcoma (21 years) In the cases described in the literature up to 1962, there was a slight predominance of men (*Dahlgren* 1962) Thus, the female male ratio was 15/19 All our cases were men

The thorothast is easily demonstrated in ordinary histological sections Haematoxylin-eosin stains it purple and van Gieson brownish-black Some previous investigators (*e g Bazerga* 1960) have also used autoradiography to demonstrate it Microradiography, which has not previously been used to study thorotrast deposition in man, has the advantage of being applicable to sections of widely varying thicknesses With the aid of microradiography stereoscopic pictures are also easily obtained Therefore microradiography will supply more accurate information concerning the volume distribution of thorotrast than autoradiography does Microradiography has also advantage that it takes only some hours to perform, whereas autoradiography takes some months

SUMMARY

Three cases of thorotrast-induced tumours of the liver are reported In contrast to the cases previously reported the doses of thorotrast injected were smaller and the induction periods were longer Microradiography has advantages over earlier methods of demonstrating thorotrast in autopsy material, because a good stereoscopic picture of the distribution is obtained

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PATHOGENESIS OF AMYLOIDOSIS

The Two-Phase Cellular Theory of Local Secretion

By

GUNNAR TEILUM

Received 20 ix 63

Previously there has been very little exact knowledge of the source or mode of formation of amyloid A favored theory relates amyloidosis to hyperglobulinaemia and it is well established that serum globulins regularly rise in experimental amyloidosis and then decline as amyloid is laid down in the tissue *Virchow* (1871) held the view that a precursor of amyloid in the circulating plasma is transformed into a gel after crossing the vessel walls *Letterer*, long a proponent of the antigen anti[illegible]

[illegible] protein precipitated in tissues by a local non-immunological reaction or by an *in vivo* antigen-antibody reaction. All experimental attempts to induce amyloidosis on these lines have consistently failed

The alternative *"two-phase cellular theory of local secretion"* has been founded on the following experimental results and observations in experimental and "secondary" amyloidosis (*Teilum* 1951, 1952, 1954, and 1956) These histochemical studies showed direct evidence that amyloid is formed *in situ* by fixed reticulo endothelial cells by local secretion of polysaccharide containing globulins which remain in juxtaposition to these cells in the form of insoluble aggregates

The significance of these new facts should be discussed in extent and integrated with observations in various types of amyloidosis in experimental conditions and natural disease

The main results of this work may be grouped as follows

1 *Effects of Cortisone, ACTH, and Nitrogen Mustard in Promoting amyloidosis*

In 1951, Teilum for the first time called attention [illegible] between [illegible]

cl [illegible]

This investigation was supported by grants from the *King Christian X Fund* and the *Danish League Against Rheumatism*

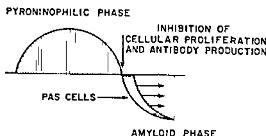


Fig 1

Diagram of cellular phases preceding amyloid formation *in loco*

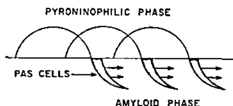


Fig 2

Diagram showing the effect of nitrogen mustard in enhancing amyloidosis in mice treated with caseinate injections

formation in mice treated in advance with injections of sodium caseinate The amyloid production was obviously associated with a *suppression* of the pyroninophilic reticulo-endothelial cells that had proliferated and differentiated along "plasmocytic" lines and concerned with protein synthesis and antibody production (Fig 1)

Continued studies on experimental amyloidosis in mice showed a similar, but even more pronounced effect of nitrogen mustard (Teilum, 1954) Three subcutaneous injections—each equivalent to 2,5 to 5 mg per kg—at intervals of two days induced rapid and extensive development of amyloidosis in the spleen, while the biopsies from the same animals before administration of nitrogen mustard only showed a very early stage of amyloid formation in the perifollicular zone of the spleen

Altogether these effects of cortisone, ACTH, and nitrogen mustard are convincing evidence of *suppression* of reticulo-endothelial cells, that have proliferated in a manner essentially similar to the normal cellular response to antigenic stimulation, to account for amyloid formation induced by various means

2 *Origin of Amyloid from Fixed PAS-Positive Reticulo Endothelial Cells* (*Teilum* 1956) (48)

Obviously, the genesis of amyloid can be studied only in the early stages, since the evidence of its mode of formation is lost in the late stages in which amyloid has lain for a long time in the tissues However, in our experimental studies, the utilization of the marked amyloid-enhancing effect of substances as cortisone and nitrogen mustard,

by which the new formation of amyloid was rapidly forced within a few days offered the opportunity to reveal a transitory appearance of reticulo endothelial cells colouring by the periodic acid Schiff (PAS) technique and directly concerned in the production of amyloid *in situ* In this transitory stage amyloid was laid down continually by new generations of reticulo endothelial cells (Fig 2) characterized cyto chemically by the presence in the cytoplasm of a polysaccharide con taining substance which was coloured by the PAS method (Figs 5 and 6) These PAS cells were appearing in rapid succession in the border zone of amyloid which already had been precipitated locally The final result was a diffuse amyloidosis with suppression of all mesenchymal cellular elements

3 *Biphasic Development* (Figs 1 and 2)

From the experimental data it appeared that the process of amyloid formation in the tissues is depending on a *biphasic* development in the protein synthesizing function of mesenchymal (reticulo endothelial) cells

a An initial *pyroninophilic* phase with proliferation of reticulo endothelial pyroninophilic cells and plasma cells and rise in serum gamma globulin

b *The amyloid phase* depending on a suppression of proliferating pyroninophilic cells and associated with a decrease in the gamma glo- bulin level

• The transition from the initial phase to the amyloid phase may follow a protracted stimulation of the immune mechanism with exhaustion of the normal process of antibody production as in natural disease or may be enhanced experimentally by administration of cortisone or nitrogen mustard The amyloid-enhancing effect of cortisone is of course depending on a pre existing initial phase with proliferating

tage of synthesis

cortisone before

final result may

... of amyloid formation or not depending on the dosage of cortisone (*Christensen* 1961)

Amyloid production is thus traced back to a cytochem ... defined ...

... of these cells in the late pyroninophilic and the early stage of intensive amyloid formation

The pyroninophilic cells are concerned with the normal process of immunoglobulin production in response to antigenic stim ... breakdown of th ...

... the general


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circulation but remain as insoluble amyloid aggregates in juxtaposition to the cells

The *clinical* manifestations of amyloidosis are largely determined by the situation of exhaustion of protein synthesis by reticulo-endothelial cells (corresponding to the experimental cortisone- and nitrogen mustard amyloidosis) associated with a transitory secretion of a glycoprotein laid down locally and forming the main component of amyloid

CONSIDERATION OF VARIOUS EXPERIMENTALLY INDUCED OR ENHANCED FORMS OF AMYLOIDOSIS IN THE LIGHT OF THE "TWO PHASE CELLULAR THEORY"

Table 1 lists types of experimental amyloidosis enhanced or induced by various means It is of special interest that the process of amyloid formation is affected by adrenal cortical hormone of cortisone type and ACTH, which are known to affect other aspects of connective tissue function and also to induce a wide-spread decrease in plasma cells and other pyroninophilic cells in hyperimmunized animals (*Teilum et al* 1950) and linked with this a suppression of the normal process of immunoglobulin production

TABLE 1

Types of Experimental Amyloidosis Linked with Inhibition of Cellular Proliferation

1 Amyloidosis enhanced by
   cortisone and *ACTH* (*Teilum* 1951–1952)

2 Amyloidosis enhanced by
   nitrogen mustard (*Teilum* 1954)

3 Amyloidosis enhanced by
   x irradiation (*Christensen & Hjort* 1959)

4 Amyloidosis in *chronic scurvy*
   (*Pirani et al* 1949)

Figs 3 8

Figs 3 and 4 Stimulated and proliferated PAS positive primitive reticular cells containing branching fibers in spleen of hyperimmunized rabbit Periodic acid Schiff stain × 450 *and* × 900

Fig 5 PAS positive reticular cells directly concerned in the local production of amyloid in the spleen Caseinate induced amyloidosis in mice accelerated with nitrogen mustard PAS stain × 450

Fig 6 Perisinusoidal aggregates of amyloid produced directly from the littoral PAS positive cells lining the sinusoids of the liver Same animal as Fig 5 PAS stain × 450

Fig 7 Spleen from patient with rheumatoid arthritis without amyloidosis Note small aggregates of reticulo endothelial cells coloured by the PAS method PAS stain × 400

Fig 8 Spleen from patient with rheumatoid arthritis complicated with amyloidosis Note the amyloid precipitation suggesting a cellular origin *in situ* and an intimate relation to reticulum PAS stain × 400

The chloroethyl amines (nitrogen mustard) are known to be toxic to cells which are in a state of active proliferation The chromosome fragmentation which is induced is characteristic to the mustard, and because of the similarity of effects with those produced by x-rays these compounds have been termed "radiomimetic".

Lesher et al (1957) showed, that mice kept under gamma-radiation during 100 to 700 days had a much higher incidence of amyloidosis than control animals In this laboratory *Christensen & Hjort* (1959) found that a sublethal dose of x-rays as whole body irradiation caused a marked acceleration of caseinate-induced amyloidosis in mice in contrast to the non-irradiated control group

The fact that administration of nitrogen mustard as well as x-irradiation enhanced the caseinate-induced amyloidosis in a remarkable way is further evidence of a secondary inhibition of cellular proliferation and disturbance of the normal synthesis of nucleic acid to account for amyloid formation

Probably a biphasic reaction of reticulo-endothelial cells in vitamin C deficiency (*Teilum et al* 1953) analogous to the failure of normal maturation of proliferating fibroblasts may provide a reasonable explanation for the experimental amyloidosis in chronic scurvy, described by *Pirani et al* (1949)

CLINICAL FORMS OF SECONDARY AMYLOIDOSIS IN THE LIGHT OF THE 'TWO-PHASE CELLULAR THEORY' (Table 2)

1 *Amyloidosis in Rheumatoid Arthritis* (not treated with steroid hormone)

In 1948 the editors of the ninth rheumatism review (*Hench et al* p 114) stated that "the association of amyloidosis and rheumatoid arthritis rarely occurs, and then only in severe cases of long duration" However, in a series of post-mortem examinations comprising 26 cases of rheumatoid arthritis and 2 cases of rheumatoid (ankylosing) spondylitis *we found amyloid changes histologically in 17 out of the 28 cases,* which were not treated with steroid hormone (*Teilum & Lindahl* 1954) Even we will not pretend that this reflects the real incidence of this complication in rheumatoid arthritis in general since the material was supplied mainly from a hospital for chronic disease and in most cases the joint disease had been of very long duration, the very high incidence in our series points to a special relationship between these two disorders The amyloid lesions were of severe or moderately severe degree in 10 cases (35 per cent) and albuminuria had been present in 45 per cent of all cases Of the 17 patients with amyloid changes 13 had albuminuria (78 per cent) Uraemia accounted for death in 25 per cent of the 28 cases The average duration of the arthropathy was 28 years in the group with severe amyloid lesions and 12 years in the group showing mild changes

TABLF 2

Clinical Types of Amyloidosis in the Light of the Two Phase Cellular Theory of Local Secretion

1 Amyloidosis in rheumatoid arthritis
(not treated with steroid hormone)

2 *Extensive amyloidosis rapidly forced by* steroid hormone therapy (case 1 and 2)

3 Rapid and extensive amyloidosis following treatment of Hodgkins disease with nitrogen mustard

4 Amyloidosis in a gammaglobulinaemia (antibody deficiency syndrome) (*Teilum* 1964)

5 Elective amyloid deposition in the stroma of a hypernephroma in the kidney following prolonged steroid hormone therapy (case 3)

Cases of amyloidosis associated with rheumatoid arthritis have later been reported in rapid succession (*Reece & Reynolds* 1954, *Gedda* 1955, *Missen & Taylor* 1956), and to day rheumatoid arthritis is probably the most common cause of amyloidosis of the "secondary" type In this disorder there is a significant correspondance between the degree of systemic disease activity and the electrophoretic serum pattern including marked increases in the gamma globulin fraction (*Bonomo*, 1957) The increase in the plasma cells of the bone-marrow has been shown by *Hayhoe & Smith* (1951)

Obviously, the prolonged reticulo endothelial hyperactivity followed by a protracted exhaustion of the cellular synthesis of protein explains the formation of amyloid in such cases of rheumatoid arthritis not treated with steroid hormone In this connection it should be remarked that in five of our cases a distinct decrease in the [illegible]

[illegible] without amyloidosis dispersed small aggregates of reticulo endothelial cells coloured by the PAS technique (Fig 7), and cases with amyloidosis in the spleen showed isolated patches of amyloid suggesting a cellular origin in loco (Fig 8) Possibly the PAS cells directly concerned with the process of amyloid formation may be identical with the reticulum cells to which the rheumatic factor has been localized by fluorescens antibody technique (*Mellors et al* 1961)

Altogether there is also in this common type of "secondary" amyloidosis pathologic clinical evidence of a *biphasic* reaction of reticulo-endothelial cells involved in the synthesis of globulins (proliferation-suppression), the latter phase being associated with a secretion of glycoprotein *in situ* reflecting the experimental findings

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[illegible] almost simultaneously with the onset of albuminuria Histochemical *studies* of the spleen revealed in some cases of rheumatoid arthritis *without* amyloidosis dispersed small aggregates of reticulo endothelial cells coloured by the PAS-technique (Fig 7), and cases with amyloidosis in the *spleen* showed isolated patches of amyloid suggesting a cellular *origin in loco* (Fig 8) Possibly the PAS-cells directly concerned with the process of amyloid formation may be identical with the reticulum *cells to* which the rheumatic factor has been localized by fluorescens antibody technique (*Mellors et al* 1961)

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2. *Rapid and Extensive Development of Amyloidosis in Association with Steroid Hormone Therapy.*

Since the amount and rate of amyloid formation induced by the usual experimental methods could be increased by the concurrent administration of cortisone or ACTH, which themselves are known to depress antibody formation, it could be expected that prolonged therapy with these substances under certain conditions would induce or enhance amyloid formation in a remarkable way.

The first case was reported by *West & Newns* (1952), who described a 29 year old patient with rheumatoid arthritis treated with 75 mg of cortisone daily for a year A biopsy before treatment showed normal liver, but at autopsy one year later the liver was enormous, and it was estimated that 85 per cent of liver,weighing 6250 g, consisted of amyloid substance Similar cases of extensive amyloidosis following treatment of rheumatoid arthritis and purulent ileo-colitis with ACTH were noted by *Frenkel & Groen* (1954). *Wegelius* (1956) described marked amyloidosis in a case of systemic lupus erythematosus of six years duration, treated with ACTH and cortisone

I have observed several cases of this category indicating a direct causal relationship to steroid hormone therapy Two typical cases are reported here.

Case 1.

57-year-old man (State Hospital, Dept. B) suffering from rheumatoid arthritis since September 1953 and since December same year treated continuously with cortisone 75 100 mg daily as well as ACTH (Acton prolongatum) 20 i u weekly At the fourth admission to hospital February 1955 the patient had developed marked proteinuria, in November same year with an excretion of ca 5 g pro die, serumprotein was 4 67 g% (normal 6 3–8 1 g%), albumin 1 54 g% (normal 4 1–5 5 g%), total globulin 3 13 g% (normal 1 6–3 2 g%) Blood urea was increasing from 63 to 115 mg% *In October 1955 the liver could be felt 10 cm below the costal margin* Biopsies of the kidney and liver (May 1956) revealed extensive amyloid changes The patient died in May 1956 only two years and nine months after the onset of the joint disease Amyloid changes were manifest 15 months after the steroid treatment was started

The *autopsy* showed apart from peptic ulceration in the stomach and marked atrophy of the adrenals a wide spread amyloidosis in the liver (*34 × 27 × 11 cm*, weight 3100 g) spleen (320 g), kidneys and adrenals

Histologically was found a most excessive amyloidosis in the liver (Fig 9), spleen and kidneys (Fig 10) that stained positive with methyl violet and Congo red.

Evidently the amount of amyloid and the rapid development in direct connection with the prolonged steroid hormone therapy pointed to a causal relationship

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Figs 9–10

Fig 9 Excessive amyloidosis of the liver rapidly forced by prolonged steroid therapy of rheumatoid arthritis 57-year-old man Case 1 van Gieson-Hansen × 120

Fig 10 Marked amyloidosis of the glomeruli in the kidney Case 1 van Gieson-Hansen × 460

Other cases of Hodgkins disease and Hodgkins sarcoma, in which administration of nitrogen mustard has been considered a factor in production of a fairly abrupt deposition of amyloid have later been reported (*Spain* 1956, *Kilburn* 1958) The possibility of an amyloid-accelerating effect of nitrogen mustard and other therapy in Hodgkins disease was again considered by *Winawer & Feldman* (1959), *Cardell* (1961), and by *Wildhack* (1956), who studied the electrophoretic changes in serum protein in 3 cases of this category

Cardell found that 4 of 8 patients with Hodgkins disease treated with cytotoxic drugs developed amyloidosis, while in 14 patients not so treated amyloidosis did not occur

Altogether such cases suggest that nitrogen mustard therapy may contribute to the rapid development of amyloidosis in cases of Hodgkins disease in accordance with the presented concept of a biphasic cellular reaction as accounting for amyloid formation In these patients there were, of course extremely active proliferation of reticulo-endothelial cells and the suppression was caused by the administration of nitrogen mustard

4 *'Agammaglobulinaemia (Hypoimmunoglobulinaemia, Antibody Deficiency Syndrome), Associated with Secondary Amyloidosis* (49)

The occurence of "agammaglobulinaemia" or hypoimmunoglobulinaemia with secondary development of amyloidosis is of greatest theoretical interest in connection with the presented theory according to which amyloid formation is linked with *inhibition* of "plasmocytoid' and other pyroninophilic cells concerned in the production of gammaglobulin (and antibodies) A report of such a case with special reference to the pathogenesis of amyloidosis will be found elsewhere (*Teilum*, 1964) (49)

5 *Rapid Development of Amyloidosis Associated with Excessive Amyloid Deposition in the Stroma of a Hypernephroma in the Kidney Following Prolonged Steroid Hormone Therapy*

The "two phase cellular theory of local secretion' also provides a reasonable explanation for the elective amyloidosis in the stroma of a growing malignant tumour in a patient with rheumatoid arthritis treated with steroid hormone in periods for seven years

Case 3

(B H 1299 62) 70 [illegible]
tre[illegible]
in [illegible]
18 [illegible]
hac[illegible]
me [illegible]
(to [illegible] globulin α_1 0 54 α_2 0 98 β 0 58 γ 1 23 g%
The patient died 5 days after admission

Case 2

28-year-old woman (State Hospital, Dept A, J 18567) Since 1961 complaints of pain, swelling of joints and febrilia with erythematous rash on the skin of the face L F test was negative The patient was treated with prednison 10 mg pro die At admission to the State Hospital June 1962 the serum albumin was low (2 34 g%) α_2 and γ-globulin increased (1 72 and 1 81 g%) On December 17th 1962 a kidney biopsy showed moderate hypercellularity and thickening of the basement membrane of the glomeruli but no sign of amyloid deposition Steroid hormone therapy had not been given for half a year when initiated again on January 4th, 1963 in doses of 10 mg prednisone × 3 pro die On February 8th, 1963 the patient had developed a marked nephrotic syndrome with an excretion of 4–5 g protein pro die and serum protein analysis showed a marked decrease in albumin increase of α_2 and fall of γ globulin Clinically it was stated that the nephrotic syndrome had set in rather acute three weeks after steroid hormone therapy was started again A liver biopsy on March 1st, 1963 demonstrated marked deposition of (metachromatic) amyloid

I have had occasion to study three additional cases of fatal amyloidosis rapidly developed during prolonged steroid hormone therapy of rheumatoid arthritis

Also *Rubens-Duval & Villiaumey* (1959) in their study on rheumatoid arthritis and amyloidosis report cases in which the hormone therapy unquestionable has accelerated the amyloid formation, and *Heller* et al (1961) say that at least one such patient with familial mediterranean fever has come to their notice who developed rapidly fatal renal failure when receiving intensive steroid treatment in another hospital These authors refrain from using steroids in this disease of man in which the appearance of amyloidosis can be predicted in a high percentage of cases

Recently further cases of this rapid and extensive form of amyloidosis have been reported, probably induced by long-term steroid hormone therapy In *West*'s series (1962) of sixteen patients treated with weekly intramuscular depot injections of prednisolone acetate for from 1 to 3½ years (mean 2½) two patients developed hepatomegaly *during* intramuscular therapy and in the Congo red test 100 per cent of the dye was absorbed

In the light of the experimental findings it seems reasonable to assume that also this rapid and extensive development of amyloidosis in human following long-term steroid therapy is determined by the various degree of *reticulo-endothelial hyperactivity* and its *secondary suppression* during the prolonged administration of hormone

3 *Rapid and Extensive Development of Amyloidosis in Association with Nitrogen Mustard Therapy of Hodgkins Disease*

Analogous to the experimentally enhanced amyloid formation in mice following injections of nitrogen mustard it has been assumed that nitrogen mustard therapy may have contributed to the extensive and rapid development of amyloidosis in cases of Hodgkins disease by depressing actively *proliferating mesenchymal cells* (*Teilum* 1954)

The *autopsy* showed besides very marked amyloidosis of the spleen the kidneys the liver and the adrenals a rounded hypernephroma (carcinoma) 8 × 6 × 6 cm in the upper pole of the left kidney The cut surface was variegated showing typical yellow and red haemorrhagic areas

Microscopic examination showed amyloid changes in the organs mentioned above The deposition of amyloid in the kidneys was mainly confined to the glomeruli which were converted to homogeneous masses and only minimal changes were found in the interstitial stroma (Fig 14) In sharp contrast to this was found an excessive and diffuse amyloidosis of the connective tissue stroma of the hypernephroma of the left kidney (Fig 11 12 and 13)

Unlike the usual finding of a scanty but highly vascularized stroma the solid cords of typical clear hypernephroma cells in all parts of the neoplasm were separated by broad bands of amyloid (Figs 11 and 12) giving a positive stain with Congo red and methyl violet Obviously, the excessive amyloid deposition in the stroma of the rapidly growing malignant tumour can not be explained by any versions of the so-called circulating precursor theories', which assume that capillary endothelial permeability to protein must be increased to permit the circulating protein polysaccharide complex to cross the vessel walls (*Kennedy* 1962) On the other hand the elective amyloid deposition in the tumour stroma is well explained by the presented two-phase cellular theory of local secretion" According to this the chain of events will be 1 Rheumatoid arthritis associated with stimulation and proliferation of mesenchymal cells (with pyroninophilia) in glomeruli of the kidney, spleen liver, and adrenals 2 The simultaneous growth of the hypernephroma will imply an active proliferation of (pyronino philic) stromal cells in the tumour, also representing an active stage in loco 3 The inhibition of proliferating pyroninophilic cells in the stroma cells of the tumour as well as in the common sites of secondary amyloidosis caused by the steroid hormone treatment has then, in addition to the marked secondary amyloidosis, resulted in an excessive and elective amyloid deposition in the tumour stroma Apparently, the amount of amyloid so rapidly produced in the tumour is depending on the rate of proliferation of reticular stromal cells in the tumour as well as the secondary suppression of the protein synthesizing function of these cells

DISCUSSION

The presented findings have led to several conclusions on basic factors in the pathogenetic mechanism of secondary amyloidosis and the experimentally induced forms following antigenic or unspecific stimulation First the histochemical studies show direct evidence that in the

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Figs 11 12

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12

transitory stage (Fig 2) in which new formation of amyloid is rapidly forced experimentally, fixed reticulo-endothelial cells containing PAS-positive material are linked with the formation of amyloid Amyloid is formed in situ by these cells in the form of insoluble aggregates between the cells (Figs 5 and 6)

A common feature in genesis of various forms of secondary and experimental amyloidosis was the preceding biphasic cellular reaction in loco *proliferation* with pyroninophilia followed by *suppression* of pyroninophilia and immunoglobulin production The second stage may be produced by a prolonged stimulation of the immune mechanism with secondary exhaustion of the normal production of immunoglobulin as in natural disease, or it may be enhanced experimentally by administration of cortisone, ACTH, nitrogen mustard or x-irradiation or other procedures, which are known to suppress mesenchymal cells which are in a state of active proliferation

Consistent with the experimental findings have been recognized *various clinical forms* of extensive amyloidosis rapidly developed following therapy with steroid hormone of cortisone type, ACTH or administration of nitrogen mustard However, it should be emphasized that the amyloid enhancing effect of these procedures is depending on a preexisting stage of active proliferation of reticulo endothelial cells *in the* same sites which have differentiated along "plasmocytoid lines accounting also for the elevated levels of immunoglobulins in the active stage This concept has made it possible to estimate and correlate factors of importance in the basic mechanism of amyloid formation in *man* according to the experimental results It also seems to clarify many of the inconsistences adhering to earlier theories First the significance of hyperglobulinaemia which previously never has been explained or understood *Dick & Leiter* (1941) stated in this connection that the direct *attempt to test* the theory of hyperglobulinaemia as a cause of amyloidosis has yielded conflicting results difficult to interpret and like other workers they found strong evidence against the simple assumption of hyperglobulinaemia as a cause of amyloidosis More indirect attempts to correlate hyperglobulinaemia and amyloidosis had not led to a *conclusive* decision *Letterer* (1949 1959) considered a *dysproteinaemia* as *the* first and humoral basis for amyloid formation The fact that globulins regularly rise in experimental amyloidosis and then decline as amyloid is laid down has led *to the* hitherto

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Figs 13 15

Fig 13 Heavy interstitial amyloidosis in hypernephroma of the kidney Case 3 cf Fig 15 Methyl violet stain × 70

Fig 14 Section from the kidney (case 3) ...

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Fig 13 Heavy interstitial amyloidosis in hypernephroma of the kidney Case 3 cf Fig 14 Methyl violet stain × 70

Fig 14 Section from the kidney (case 3) showing marked amyloid changes in glomeruli but no involvement of the stroma For comparison with section (Fig 13) from the hypernephroma in the same kidney Methyl violet stain × 70

most widely held view of a circulating precursor It is evident that although hyperglobulinaemia and dysproteinaemia often are found in conditions associated with amyloidosis, amyloid formation is not caused by the hyperglobulinaemia *per se*, but depending on alteration of "plasmocytoid" cells involved in the production of serum gamma globulin and antibodies in response to the prolonged stimulation of the immune mechanism The suppression or exhaustion of the cellular proliferation will lead to production and precipitation of glycoprotein *in situ*, the successive *involvement of various organs being determined* by the stage of cellular synthesis of protein locally in the tissues

The observations of rare cases of hypoimmunoglobulinaemia (antibody deficiency syndrome) with secondary development of amyloidosis is further evidence that elevated levels of serum gamma globulin is not an essential factor in the fundamental pathogenetic mechanism of amyloid formation On the other hand such cases are well explained by the two phase cellular theory In most cases of agammaglobulinaemia with antibody deficiency syndrome is found a marked decrease rather than a total deficiency of gamma globulin The recurrent pyogenic or chronic infections connected with the marked decreased ability of antibody production would be expected to produce a most incomplete cellular response to antigenic stimuli Actually the occurrence of amyloidosis in such rare cases reflects the experiments in which cortisone, which suppress antibody production, and caseinate stimulating the immune mechanism, are administrated simultaneously

Analogous to this mechanism of amyloid formation in hypoimmunoglobulinaemia the presented experimental and clinical data also explain the observation (*Letterer* (1949), *Ott & Schneider* (1951)) that experimentally amyloid is laid down when the production of antibodies decreases, while a good formation of antibodies is not found associated with amyloidosis under the same experimental conditions

Obviously, the remarkable amyloid-accelerating effects of cortisone, *nitrogen mustard and x-irradiation affecting cellular proliferation* strongly support the concept of cellular changes in loco and are difficult to reconcile with the theory of a circulating precursor Apart from the direct histochemical evidence of local precipitation from PAS-positive cells, it seems most unlikely that an increased capillary endothelial permeability to protein should account for the deposition within a rather short period of several kg of amyloid in the liver or for the elective deposition in the stroma of a growing hypernephroma of the kidney following prolonged steroid hormone therapy (case 3) Also cases of local amyloid formation and studies on spleen shielding in experimental amyloidosis accelerated by x-irradiation (*Christensen & Hjort* 1960) point to the significance of a cellular synthesis and deposition in situ

Clausen et al (1960) studied the immuno-electrophoretical changes in amyloidosis experimentally induced in mice by daily injections of

caseinate or by inoculation of certain transplantable reticulosarcoma inducing amyloid First hyper immuno globulinaemia was observed the beta 2 III and the gamma beta 3 II—beta 3 III being considerably increased in concentration the beta 2 III in mobility also This stage corresponds to the proliferative pyroninophilic phase of the reticulo-endothelial cells Later increased concentration and mobility of alpha 2 I and possibly alpha 2 II was observed corresponding to the stage of amyloid formation in the spleen and liver From serological and biochemical points of view the finding of unspecific hyperimmunoglobulinaemia were in correspondance with the presented theory

When it has been claimed (*Kennedy* 1962) that the littoral cells of the liver alias Kupffer cells are not related topographically or functionally to amyloid deposition this is clearly contradicted by my histochemical observations in caseinate induced amyloidosis (*Teilum* 1952 & 1956) which have also been reproduced in later studies (*Christensen & Rask Nielsen* 1962) In all cases it was evident that the perisinusoidal deposits of amyloid were produced directly from these cells lining the sinusoids (Fig 6) It has also been objected that there is no indication of a proliferation and transition of Kupffer cells in the liver or of the cells in the glomerular tufts of the kidney to immature or mature plasma cells However it should be noted first that a proliferation of these cell types *e.g* in response to persistent antigenic stimuli has been shown to be associated with a marked pyroninophilia which only can be interpreted as evidence of increased protein synthesis by the cells This represents a basic cellular reaction preceding formation of hyalin as well as amyloid related to a hyperglobulinosis localized in the glomerular lesions in human and experimental glomerulonephritis and disseminated lupus erythematosus in the surroundings of granulomas in sarcoidosis containing hyalin material as well as the "onion skin fibrosis of disseminated lupus erythematosus and in amyloid lesions (*Teilum* 1948 & 1952) Several years later human gammaglobulin has been identified in exactly the same sites in the same conditions by the fluorescent antibody technique (*Mellors et al* 1957 *Vazquez & Dixon* 1957)

As to the reticulo endothelial cells lining the liver sinusoids reference should also be made to the extreme development of ergastoplasm lamellas and abundance of endoplasmic reticulum during amyloid formation shown by electron micrography (*Caesar* 1960 and unpublished studies by *Hjort & Christensen* in our laboratory) *Levine et al* (1962) demonstrated the presence of human globulin in the Kupffer cells as well as in the deposits of amyloid in a patient with primary systemic amyloidosis Also the small dark reticulum cell comparable with Marshall's fixed syncytial metalophil cell developed an ergastoplasm after antigen stimulation and was considered to have a limited ability of antibody production (*Christensen* 1960) As facts remain that the process of local formation of amyloid is not restricted to cells showing the

morphological characteristics of plasmocytes. Although there is convincing evidence that the insoluble aggregates of amyloid of the common secondary type of amyloidosis as well as the experimental forms induced by caseinate injections are linked with a perversion of the process of antibody production and in such cases have a cellular origin in common with the immunoglobulin there may be several exceptions, also in the experimentally induced forms. The biphasic pyroninophilic reaction observed in experimental scurvy (*Teilum et al.* 1953) may account for the development of amyloid in scorbutic animals as described by *Pirani et al.* 1949. Evidently, different types of proliferating mesenchymal cells, including young and immature plasma cells, cells of the glomerular tufts, the lining cells of the sinusoids of liver and adrenals, and primitive reticular cells of tissue interstices (cf. case 3) may be involved and irrespective of varying ability for specialized differentiation towards veritable antibody production, the phasic development regarding protein synthesis and the fundamental characteristics of the pathological process are broadly the same throughout. The course of these cellular changes leading to the local precipitation of amyloid is determined by the influence of conditioning extrinsic or intrinsic factors, such as adrenocorticoids, the effect being dependent on differentiation and phase in protein-synthesizing function of the cells in loco. Various factors of cellular inhibition connected with suppression of antibody production and accentuating the second stage of amyloid formation have already been listed. It is of interest that these factors closely correspond to causal factors in hypogammaglobulinaemia and antibody deficiency syndrome considered by *Barandun et al.* 1959 in their comprehensive study of this disorder, such as massive doses of corticosteroids and ACTH, nitrogen mustard, x-irradiation and C-avitaminosis.

Variability in staining reactions suggests that amyloid is not a uniform chemical substance, but a series of closely related protein compounds, the composition of which may vary from one case to another and even in different regions within the same case. The smaller component of metachromatic material possibly originates from endothelial cells (22, 11) or from metallophilic reticular cells (11).

Other Forms of Generalized Amyloidosis

Familial Mediterranean Fever is a genetically determined disease of man in which the appearance of amyloidosis can be predicted in a high percentage of cases. The fact that also amyloidosis in this disorder may be enhanced by treatment with corticosteroid hormone (*Heller et al.* 1961) points again to a common factor of cellular inhibition in different forms of amyloid disease.

Primary Systemic Amyloidosis (paramyloidosis) and the Related Condition Complicating Myelomatosis

There are fewer histochemical and experimental studies of the paramyloid infiltrates that develop in association with plasma cell myeloma and the related condition of primary systemic amyloidosis or paramyloidosis. The infiltrates in these cases are mostly found in atypical "non reticulo-endothelial" sites in the mesenchymal tissue, such as walls of vessels surrounding cardiac, skeletal and smooth muscle cells and giving rise to characteristic amyloid rings around fat cells, which are often found in the periadrenal adipose tissue. The commonly held view that the neoplastic myeloma cell as such is the direct source of of the paramyloid infiltration at these sites (*Apitz* 1940) or that an increased capillary permeability to protein should account for the unique selection of certain organs and tissues (*Osserman* 1959, 1961) should be doubted.

From the represented studies it will appear that formation of amyloid, also of the secondary type, is not restricted to mesodermal cells of "plasmocytic" type. Also the lining cells of the sinusoids in the adrenal cortex are cells of the macrophage system, much like those lining the sinusoids of the liver and hypophysis, and the interstitial connective tissue are of reticular type containing branching fibers (Figs. 3 and 4) and have marked mesenchymal potencies to develop into other type of connective tissue cells. Such mesodermal stromal cells, which are stimulated and abnormally proliferated and diffentiated under the influence of similar causal factors may be the source of amyloid formation at the same sites. This is also substantiated from the observations in case 3 of rapid and excessive formation of amyloid in the interstitial stroma of a growing renal carcinoma (hypernephroma) following intense steroid hormone treatment, which simultaneously accelerated the formation of secondary amyloid in the common, "reticulo-endothelial" sites in rheumatoid arthritis. It is also worthy of note that ... [illegible]

... as basis for amyloid formation in tumour stroma and the reticuloendothelial organs is produced in different ways and connected with the neoplastic growth and rheumatic disease respectively, the secondary phase with inhibition of cellular proliferation and resulting in excessive amyloid formation was in either case provoked by the intense steroid hormone administration. The striking discrepancy between the excessive formation of amyloid in the stroma of the tumour and that of the interstices of the remaining tissue of the kidney, (cf. Figs. 13 and 14) again demonstrates the decisive significance of the initial proliferative phase in the fundamental mechanism of amyloid formation.

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manifestation of mesenchymal tissue dysfunction (*Rotstein & Good* 1962)

Previously it was found that primary and secondary amyloidosis exhibited similar changes in serum protein (*Calkins & Cohen* 1959) While paraproteinaemia seems to occur mainly in myelomatosis and Waldenstroms macroglobulinaemia recent immunoelectrophoretic studies of *Clausen & Christensen* (1964) revealed in all of four investigated cases of primary systemic amyloidosis with involvement of heart and tongue a gammaparaproteinaemia associated with a hypoimmunoglobulinaemia

In a review on primary amyloidosis *Symmers* (1956) stated that no other criterion than the absence of a recognizable predisposing cause is needed in order to designate any case of amyloidosis as primary amyloidosis Even though this seems logical studies on immunoelectrophoretic pattern may possibly justify a distinction between primary cases with atypical distribution of paramyloid and cases showing the classical distribution although no predisposing cause is recognized While there is no reason to assume that cases of primary systemic amyloidosis (paramyloidosis) are actually cases of occult plasma cell myeloma the common distribution and composition of the amyloid aggregates in these two conditions may point to similarities in the pathogenetic mechanism Also it seems unlikely that the mode of formation and basic cellular error in the non reticular and the secondary types of amyloidosis should be quite different even the ways in which the disturbance in cellular protein synthesis is provoked may vary in the different forms In this connection the general significance of a hypoimmunoglobulinaemia as a manifestation of mesenchymal tissue dysfunction should be considered

To sum up the experimental findings integrated with the observations in various clinical forms of amyloidosis in man would [illegible]

[illegible] mesenchymal cells under the stress of prolonged stimulation or hyperimmunization Considering the substantiated fundamental features in the second phase of amyloid formation and those in cases of hypoimmunoglobulinaemia (as reflected by the same family of cells related to the production of serum gamma globulin and antibodies wh[illegible]

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basic cell[illegible] [illegible] conditions may be very similar The perversion in the local cellular synthesis of protein leading to amyloid formation could be due to a disturbance of some enzyme system (*Teilum* 1952) possibly some unrecognized inhibitor producing a metabolic block or a structural defect in plasma cells and its precursor as suggested by *Martin* (1962) as the cause of the basic lesion in hypogammaglobulinaemia

duced type generally is preceded by an hyperimmunoglobulinaemia in the initial phase and the gradual exhaustion and depression of cellular protein synthesis may lead to a local precipitation associated with a hypoimmunoglobulinaemia The mechanism by which a similar disturbance in protein synthesis of reticular cells is induced accounting for the formation of paramyloid aggregates in cases of primary systemic amyloidosis and the related condition complicating myelomatosis is unknown and yet the basic cellular reaction seems in principle to be similar in the primary and secondary form

Analyses of the serum globulin levels have shown, contrary to what should be expected according to the the theory of *Apitz* (1940), that hyperglobulinaemia is rare in patients with myeloma who develop amyloidosis (*Eisen* 1946) This was also found in the series of *Snapper et al* (1953) Of their eleven myeloma patients with paramyloidosis only three had a serum globulin above 2 5 per cent Interestingly, however, Eisen drew attention to the fact that in several cases of myeloma with amyloidosis there was hyperglobulinaemia earlier in the disease, with globulin falling later, in contradistinction to uncomplicated myeloma with hyperglobulinaemia often persisting to death It should also be noted that cases of myelomatosis often are connected with an immunologic abnormality considered to be the result of abnormal function of the malignant plasma cells whereby deranged protein synthesis results in production of abnormal proteins at the expense of normal proteins, including antibody protein (*Lawson et al* 1955) The electrophoretic peaks of gamma-globulin thus do not indicate an increase of functional antibody Increased susceptibility to infection is characteristic in this disorder resulting from the fact that these patients exhibit a poor antigen-antibody mechanism Also *Young et al* (1955) stated that hypogammaglobulinaemia is not uncommonly seen in myelomatosis with a β-globulin or M-component abnormality, and may well be due to division of gammaglobulin precursors for the synthesis of the abnormal components Such a patient had a history of many bouts of sepsis, particularly pneumonia, since the onset of the disease He failed to develop antibodies in response to typhoid vaccination and the tuberculin and histoplasmin skin tests were negative While secondary amyloidosis may have a near relation to rheumatoid arthritis (*Teilum & Lindahl* 1954), the primary systemic amyloidosis or paramyloidosis shows many features in common with other of the so called collagen diseases (*Teilum* 1948) as manifest by the same and wide-spread distribution with involvement of the walls of small blood vessels and mesenchymal tissue of the heart, gastrointestinal tract, skin, peripheral nerves and periarticular tissues, as in generalized scleroderma, and the associated changes in synthesis of serum globulins, characteristic for several members of the group Dermatomyositis juvenil rheumatoid arthritis and scleroderma have been reported in agammaglobulinaemic patients in the literature and agammaglobulinaemia is considered an overt

in a case of secondary amyloidosis following long-term steroid hormone therapy in rheumatoid arthritis, strongly supports the presented theory of local secretion and is incompatible with the assumption of increased capillary endothelial permeability to account for amyloid deposition

It is concluded that the common fundamental lesion in various forms of amyloidosis is a failure of normal differentiation and maturation of plasmocytoid and other pyroninophilic reticular cells under the stress of protracted stimulation, the process being determined by ex- or intrinsic factors, such as corticosteroids and dependent on the differentiation and the phasic stage in the protein synthesizing function of the cells *in loco*

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The cellular defect may be *genetic* and possibly accounting for familial forms of amyloidosis or it may be *acquisit* under the stress of prolonged antigenic or unspecific stimulation or accelerated by administration of such substances as cortisone, ACTH or nitrogen mustard, which are known to suppress mesenchymal cells which are in a stage of active proliferation It may also be induced by x-irradiation and in chronic scurvy and not necessarily associated with an immunologic phenomenon

It is believed that the consideration of cellular phases in the reticulo-endothelial tissue, related to hyperimmunization and affected by substances, which are known to affect other aspects of connective tissue function, may contribute to the clarification of many of the inconsistences adhering to earlier theories and help to open a new field of investigation in relation to amyloidosis and other manifestations of mesenchymal tissue dysfunction

SUMMARY

The pathogenesis of amyloidosis is discussed in the light of "the two phase cellular theory of local secretion" and integrated with observations in various types of amyloidosis in experimental conditions and natural disease

The process of amyloid formation in the tissues is depending on a biphasic development in the protein-synthesizing function of pyronino philic reticulo endothelial cells In the stage of amyloid formation following suppression of proliferating cells, amyloid is laid down continually *in loco* by new generations of cells characterized cytochemically by the presence in the cytoplasm of a substance colouring by the PAS-method

These PAS positive cells are the primary source of amyloid formation

Previously, the rôle of hyperglobulinaemia in the pathogenesis of *amyloidosis has never been understood The presented findings do* not support any versions of the widely held theory of a circulating precursor of amyloid Hyperglobulinaemia *per se* is certainly not a cause of amyloidosis The decrease in plasma globulin noted as amyloid deposition occurs is secondary to the inhibition of protein-synthesizing function of cells

Special attention should be made to the factor of *inhibition* of cellular proliferation *in loco* in the stage of amyloid formation as manifest by the amyloid-enhancing effect of substances as cortisone, ACTH and nitrogen mustard in experimental conditions and in various clinical types of amyloidosis is well as by the observation of amyloidosis in a case of agammaglobulinaemia

Also the finding of elective and intense amyloid formation in the stroma of a rapidly growing carcinoma (hypernephroma) of the kidney

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SOME EFFECTS OF FOLIC ACID ANTAGONISTS ON VIRUS INDUCED CHICKEN ERYTHROLEUKAEMIA

By

BJORN STENKVIST and JAN PONTEN

Received 15 x 63

Most experimental work on the effects of cancer chemotherapeutic agents has been carried out with transplantable tumours Although this has obvious practical advantages the response of a transplantable tumour may differ significantly from that of a primary neoplasm The only primary tumours that approach transplanted tumours from the point of view of predictable behaviour and rapid induction seem to be the virus-induced avian sarcomas and leukaemias

This paper reports the results of treatment of virus-induced chicken erythroleukaemia (erythroblastosis) by folic acid antagonists Special attention was given to a comparison between treatment instituted early or late and to differences in the response of leukaemias induced by different amounts of virus

No systematic studies of the effects of folic acid antagonists on virus-induced erythroleukaemia seem to have been published Some work has, however, been carried out with the Rous sarcoma—another member of the avian leucosis complex *Little et al* (1) demonstrated a lowered incidence of Rous sarcoma after treatment with a folic acid antagonist *Ringsted* (2) showed that aminopterin prolonged the time between virus inoculation and appearance of tumour without affecting the growth rate of the tumours *Engelbreth Holm et al* (3) found that the growth rate of Rous sarcoma was diminished by dietary deficiency in folic acid

MATERIALS AND METHODS

Animals Non inbred White Leghorn chickens from a commercial source (Edo) were used

Haematologic methods These have been described previously (4)

*Folic acid antagonists** Aminopterin (4 aminopteroylglutamic acid) or methotrexate (4 amino N^{10} methylpteroylglutamic acid) was used The drugs were given intraperitoneally dissolved in distilled water A fresh solution was made up on each day when treatment was given

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This work was supported by a grant from *Statens Medicinska Forskningsråd*
We wish to thank *Miss Marianne Andersson* for most valuable technical assistance
* We are indebted to American Cyanamid Lederle for the drugs

Virus Chicken erythroblastosis virus (CEV) was prepared according the following method previously described for purification of Rous sarcoma virus by *Gessler et al* (5)

Spleens from chickens with advanced virus induced erythroblastosis were pooled and minced with scissors. Four grams of the minced tissue was homogenized for ten minutes in 10 ml of trifluorotrichlorethane (CCl_2F $CClF_2$ Genesolv D Allied Chemical New York, N Y) and 20 ml of McIlvaines citrate buffer (pH 7.4 diluted 1 50) in a VirTis homogenizer set at 20 000 r p m. The homogenate was then centrifuged at 2000 r p m for five minutes after which three distinct layers formed. The middle layer was kept and the remaining two layers were mixed with 20 ml of McIlvaines citrate buffer and homogenized and centrifuged as described above. The middle layer which formed after this procedure was combined with the first middle layer and with 10 ml of Genesolv. After homogenization and centrifugation of this mixture two layers formed. The clear top layer had a volume of 29 ml and contained the purified virus.

Bioassay of Chicken Erythroblastosis Virus

Virus activity was determined by bioassay in 14 days old chickens. Serial ten fold dilutions were inoculated intravenously in the test animals. 5 birds were used for each dilution in the titrations of the purified virus and 40 birds per dilution were used when the virus content of leukaemic bone marrow was determined.

RESULTS

Purification of CEV

It is seen from Table 1 that the virus activity calculated on a protein nitrogen basis had increased in the fluorocarbontreated material. No takes were seen in the control birds which were inoculated with 2.0×10^{-3} mg or less protein nitrogen as compared to 3/5 takes in the group that received inocula of the fluorocarbontreated material containing 1.3×10^{-3} mg of protein nitrogen. This corresponds at least to a one hundred fold purification of the virus.

TABLE 1

Bioassay of Chicken Erythroblastosis Virus Activity in Homogenates of Leukaemic Spleens Treated With Fluorocarbon

Fluorocarbon treated suspension		Control suspension	
mg protein-N per dose	Fraction leukaemic birds	mg protein N per dose	Fraction leukaemic birds
1.3×10^{-3}	3/5	2.0×10^{-3}	0/5
1.3×10^{-2}	2/5	2.0×10^{-2}	0/5
1.3×10^{-1}	1/5	2.0×10^{-1}	0/5
1.3×10^{0}	4/5	2.0×10^{0}	2/5

The Susceptibility of Chickens of Different Age to Aminopterin and Methotrexate

Groups of chickens of different age were inoculated with various amounts of aminopterin as indicated in Table 2. Erythrocyte and

TABLE

Effect of Aminopterin and Methotrexate on Chickens of Different Age

Chicken age (days)	No animals	Dose mg kg and day	Folic acid antagonist	Duration of treatment (days)	Dead per cent
2	12	0 2	aminopterin	38	100
2	12	0 4	"	38	100
2	9	0 8 (1 0)	"	38 (48)*	89
21	12	0 2 (1 0)	"	9 (19)§	0
32	12	0 3	"	14	0
32	12	0 4	"	14	0
32	12	0 6	"	14	0
2	12	3	methotrexate	18	75
2	11	6	"	18	100

* After 38 days of treatment interval to day 41 whereafter the daily dose was raised to

§ After 9 days of treatment interval to day 13 whereafter the daily dose was raised to

granulocyte counts were done at approximately weekly intervals As seen from the table there was considerable decrease in susceptibility with age 2 days old chickens showed 100 per cent mortality when given 0 2 mg aminopterin per body weight per day as compared to no mortality in a group of 12 chickens given 0 6 mg per kg body weight per day when 32 days old

Depressing effects on the granulocyte and erythrocyte counts were seen in groups where there was some mortality Presumably due to the shorter life span of the granulocytes they were affected at an earlier point of time than the erythrocytes

Table 2 also illustrates the fact that methotrexate was less toxic than aminopterin since there was only a mortality of 75 per cent after daily treatment with 3 mg of methotrexate, whereas even such a low dose as 0 2 mg of aminopterin led to a mortality of 100 per cent

The Efficiency of Short Term Treatments of Erythroleukaemia Started at Different Points of Time after the Inoculation of Virus

117 chickens, 19 days of age, were inoculated with a high dose of CEV They were divided into five groups, one of which was left without any treatment The remaining four groups were given 0 3 mg aminopterin per kg body weight per day during a five day period In relation to the day when virus was inoculated, treatment was started on the day before one day after, four days after or seven days after As seen from Table 3, the mean survival time in group I, which recieved no treatment was 10 8 days, which was not statistically different from the figures for group IV and V where treatment was started four or seven days after inoculation of virus In group II, where the five day period of aminop-

lics Indicate Significantly Changed Blood Values

Amount of erythrocytes and granulocytes mm³ blood in per cent of normal starting values Mean values																	
Days after onset of treatment																	
2		8-9		13-14		16-1		19-21		28		31		42		45	
ery	gra	ery	gra	ery	gra	ery	gra	ery	gra	ery	gra	ery	gra	ery	gra	ery	gra
101	*87*	*89*	*92*	*98*	*91*												
97	77	92	64	81	83	67	35										
97	67	100	57	77	47	86	49	83	*31*	90	77	95	93	95	113	78	68
93	85	88	75	92	97			91	119								
92	102			97	87												
100	91			94	110												
100	107			93	90												
93	81	93	77	99	91	98	104	94	81	94	54	104	107	89	100	86	81
100	*60*																

0 mg until day 49 when treatment was terminated
0 mg until day 20 when treatment was terminated

term treatment was started on the day *before virus was given and in group III*, where treatment was started the day after virus was given, there was a statistically significant prolongation of the survival period *to 19 1* and 14 5 days respectively

If the number of lethal leukaemias was *considered the results* were *most* apparent in group II, where treatment was started already on the day before CEV was inoculated Only 13 24 animals succumbed *to* leukaemia as compared to 25/25 in the untreated *control group* 5 *animals* survived in spite of the *presence of* erythroblasts in smears of the peripheral blood Since no relapses into leukaemia ensued after the treatment was *discontinued*, these figures mean that it was possible to achieve a *permanent* cure to virus induced chicken *erythroleukaemia*

The observation time for the negative surviving *animals* was 52 days

The Effect of Aminopterin on Erythroleukaemias Induced by Different Amounts of Virus

It has been observed (4) that *erythroleukaemias* induced by a low infectious virus dose tend *to be more* differentiated resembling "chronic" leukaemias rather *than* the undifferentiated "acute" types seen after moderate and high doses of virus It was therefore of interest to investigate whether these *two* types of leukaemias would *respond* differently to folic acid antagonists

160 chickens, 19 days *old* at the beginning of the experiment were inoculated with four tenfold *dilutions* of CEV For each dilution the animals were divided into two groups of twenty birds, one of which was treated with *aminopterin* and one left as untreated controls Aminopterin treatment was started on *the day after* virus inoculation

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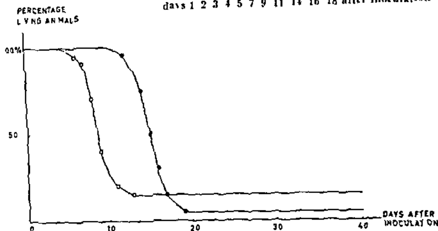


Fig 1

The effect of methotrexate on erythroleukaemia induced by an undiluted CFV preparation

Since methotrexate was less toxic than aminopterin (Table 2) the experiment was repeated using methotrexate rather than aminopterin

120 four weeks old chickens were given virus of two different concentrations differing by a factor of 1000 40 animals received the higher dose and 80 the lower Both groups were divided into two equally large subgroups one of which was treated with methotrexate and one was given no treatment

Figure 1 gives the result of treatment of the group inoculated with the largest dose of virus It is seen that methotrexate had a considerable effect on the survival time In the non treated group 50 per cent of animals were dead on day 9 whereas 50 per cent mortality was not reached until day 15 in the treated group It is noteworthy that there

e animals given a 1000 fold
... than in Figure 1 have been plotted It is seen that the effect of treatment was less pronounced than in Figure 1 There was only a small prolongation of the survival time and no effect on the final mortality

The Presence of Virus in the Bone Marrow of Untreated and Aminopterin Treated Erythroleukaemias

Virus titrations were made from the bone marrow of ten chickens selected at random from groups I III of one of the previous exper

TABLE 3

Effect on Virusinduced Erythroleukaemia of 5 Days of Aminopterin Treatment Administered at Different Time during the Development of the Leukaemia Dose 0 3 mg Aminopterin/kg Body Weight/Day Chicken Age at the Time of Virus Inoculation 19 Days

Group number	Treatment period	Number animals						Mean survival time (days)
		Total	Leukaemic blood picture	Lethal Leukaemia	Remissions	Dead of treatment	Negative surviving	
I	Untreated	25	25	25				10 8
II	—1 to +3	24	18	13	5		6	19 1*
III	+1 to +5	25	21	21		4		14 5*
IV	+4 to +8	23	16	16		5	2	11 0
V	+7 to +11	20	13	15	3		2	11 0

* These values are significantly separated from the untreated controls No significant differences between group II and III

and continued during 16 days The animals were observed over a period of 60 days

Treatment with aminopterin lowered the mortality in the group inoculated with the largest amount of virus from 100 to 74 per cent Also in the group with the next largest virus dose there was a decrease from 78 to 55 per cent Statistical analysis of the figures from these two groups showed the decrease in the mortality to be significant ($0 02 > P > 0 01$) The prolongation of the survival time, 4 6 and 14 2 per cent in the respective groups, was statistically probably significant ($0 05 > P > 0 02$)

In the third group, in contrast, there was no significant effect of the treatment with aminopterin on the mortality or the mean survival time (Table 4)

TABLE 4

Effect of Aminopterin on Erythroleukaemia Induced with Different Virus Doses 0 2 mg Aminopterin/kg Body Weight per Day during 16 Days with Beginning the Day after Virus Inoculation

Group	Relative virus dose	Aminopterin treatment	Number of animals	Accidentally dead	Cases of Leukaemia	Leukaemia per cent	Mean survival time in days
I	1000	—	20	0	20	100	11 9
	1000	+	20	1	14	74	12 4
II	100	—	20	2	14	78	13 0
	100	+	20	0	11	55	14 4
III	10	—	20	0	4	20	16 2
	10	+	20	2	5	28	15 3
IV	1	—	20	1	0	0	
	1	+	20	0	0	0	

* Correction made for accidentally dead animals

duced by low doses of CLV observed here may therefore have been due to the more differentiated character of these leukaemias This has an interesting counterpart in findings with human leukaemias since it has been shown that folic acid antagonists treatment is much more effective in giving remissions in acute than chronic leukaemias (6) in human patients

The virus content of leukaemic bone marrows did not diminish under the influence of aminopterin It is therefore improbable that the folic acid antagonists had any major effects on the synthesis of virus *per se* their effect is probably best explained as an inhibitory effect on proliferating immature erythroblasts *Since virus probably plays* an important role (7) for sustained progressive leukaemic disease in chicken is is possible that the relative failure of the folic acid antagonists to accomplish permanent cures may be due to their inability to suppress virus synthesis Even in the presence of a folic acid antagonist virus is released and continued new infection occures Drug resistance in a virus yielding tumour therefore may not only be explained by mutation and selection among the cells but by the failure of the drug to inhibit continued new infection by virus

SUMMARY

Chicken erythroleukaemia (erythroblastosis) has been treated with the folic acid antagonists aminopterin (4 aminopteroylglutamic acid) and methotrexate (4 amino N[10] methylpteroylglutamic acid)

The leukaemias were induced by a purified virus A 100 fold purification *on a protein* nitrogen basis was obtained by the use of fluorocarbon

To *obtain* any *effect* on leukaemia treatment had to be instituted early *i e not later than 4 days* after inoculation of virus

The effect *of the drugs* was manifested as a decreased mortality and a prolongation of the *survival time* The effects were most obvious in leukaemias induced by *high virus doses The leukaemias* induced by low virus doses *were to* a large extent refractory to treatment

No differences in virus content of the bone marrow could be demonstrated when a *comparison* was made between treated and untreated leukaemias

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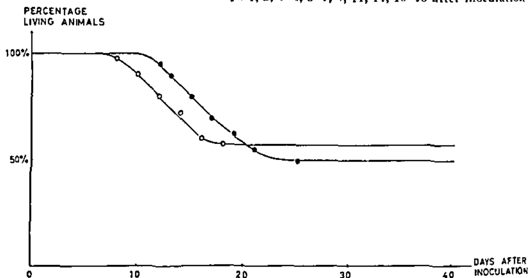


Fig 2

The effect of methotrexate on erythroleukaemia induced by CEV diluted 10^3 times (The undiluted virus was the same as that used in Fig 1)

iments (Table 4) The bone marrow from animals dying from leukaemia was removed, homogenized and virus was extracted using fluorocarbon (5) The virus was bioassayed as described above Virus could be demonstrated in all extracts There were no significant differences in titre between the different samples

DISCUSSION

Virus induced chicken erythroleukaemia proved to be a convenient tool in exploring certain aspects of the effects of treatment with folic acid antagonists It was possible to obtain a significant prolongation of the survival time and in some experiments apparently also an increased number of permanently surviving birds provided treatment was started early

A connection between the degree of maturity and aminopterinsensitivity in normal rat bone marrow cells has been demonstrated by *Reizenstein* (8) Only immature cells responded to aminopterin by cytochemical changes, mitotic disturbances and inhibition of maturation, whereas more mature cells seemed to be relatively unaffected *Ponten* has shown (4) that by infection with a low virus dose, it was possible to obtain more differentiated "chronic" leukaemias than with high doses of CEV, which gave rise to "acute" undifferentiated leukaemias The poor effects of folic acid antagonists on leukaemias in-

duced by low doses of CEV observed here may therefore have been due to the more differentiated character of these leukaemias. This has an interesting counterpart in findings with human leukaemias since it has been shown that folic acid antagonists treatment is much more effective in giving remissions in acute than chronic leukaemias (6) in human patients.

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The effect of the drugs was manifested as a decreased mortality and a prolongation of the survival time. The effects were most obvious in leukaemias induced by high virus doses. The leukaemias induced by low virus doses were to a large extent refractory to treatment.

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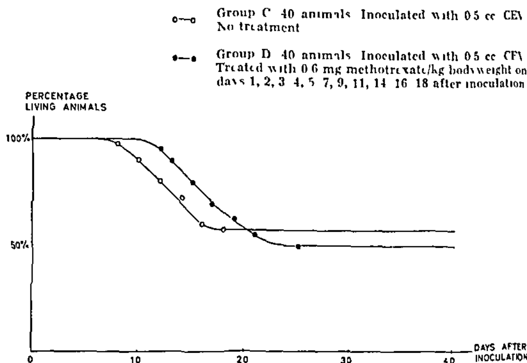


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THE EFFECT OF ADRENALINE ON THE MITOTIC RATE IN THE EPIDERMIS OF HAIRLESS MICE

By

ARNE EVENSEN

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It has been maintained that adrenaline has an antimitotic effect (*Bullough* 1955, *Gelfant* 1960, *Bullough & Laurence* 1961). It seems unlikely, however, that adrenaline which speeds up so many physiological processes should inhibit the process of mitosis. *Evensen & Heldaas* (1964) have demonstrated that great doses of adrenaline immediately increase the epidermal cell proliferation *in vitro*, and *Kreyberg & Evensen* (1964) have shown that the mitotic duration decreases when the animals are most active, and increases during rest and sleep. *Selye* (1950) maintains that nervous stimuli, among them intense light or sound, provoke the "alarm reaction". This reaction provokes an almost immediately discharge of adrenaline from the adrenals into the blood. Somewhat more slowly, but still during the first hour of the "alarm reaction", the adrenalin cortex begins to discharge its stores of hormones into the blood to meet the urgent demand. During the "alarm reaction" the animals are immediately and during the first hour under the influence of adrenaline. Later, when the stores of adrenaline are emptied, the animals are mostly under the influence of corticoid hormones.

Experiments have been performed to study the immediate and the prolonged effect of adrenaline injections on the epidermal mitotic rate of hairless mice. As the effect of a single injection of adrenaline is of short duration only, the prolonged effect of adrenaline has been studied by repeated injections of the hormone during the experimental period.

EXPERIMENTS AND RESULTS

In the experiments the strain of hairless mice of this institute has been used (*Iversen & Evensen* 1962). The animals used in each experiment have been of the same age, weighing between 20 and 24 g, and the animal groups have always consisted of an equal number of males and

This work has received financial support from *Elisabeth & Knut Knutsens O. A. S. fond for kreftforskning* and *Grosserer N. A. Stangs legat til kreftsykdommers bekjempelse*.

5 *Gessler A E*, *Bender, C E* & *Parkinson M C* A New and Rapid Method for Isolating Viruses by Selective Fluorocarbon Deproteinization Trans N Y Acad Sci Ser 2 701–703 707–716 1956
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2 the number of mitosis was 2 2 ± 0 13 per cent of the nucleated epi dermal cells These results demonstrate obviously that 0 1 mg of colcemid does not arrest the epidermal mitosis for five hours in these animals when they are under influence of high doses of adrenaline (see Table 2)

TABLE 2

The Prolonged Effect of A lrenal ne on the Fp lermal Mitol c Rate In Colcem l Treated Animals

	Number of animals	Mean per cent of mitoses arreste l with colcemi l
Controls	4	2 2 ± 0 13
10 micr g adrenal ne during f ve h urs	4	1 24 ± 0 12

Experiment No 3

The immediate effect of adrenaline on the epidermal cells in the G₂ phase (the premitotic resting phase) Two groups of four animals each were injected intraperitoneally with 10 microcurie tritiated thymidine in 0 1 ml saline solution 15 minutes later the animals were injected with 0 1 mg of colcemid in 0 25 ml saline solution Shortly after the injection of colcemid group no 1 was injected intraperitoneally with 10 microgrammes of adrenaline in 0 1 ml saline solution Group no 2 served as controls Both groups were killed one hour and 15 minutes after the injection of tritiated thymidine and one hour after the injection of adrenaline in group no 1

The stripping film technique was used for autoradiography the film was exposed to the histological sections for 14 days and after developing and fixing of the film the histological sections were stained with haemotoxylin throug the film emulsion Labelled cells labelled mitoses and unlabelled mitoses were scored among 2 000 nucleated epidermal cells in each animal The results are shown in Table 3 and demonstrate that the immediate effect of adrenaline on the epidermal cells in G₂ phase is to give them an impetus to enter mitosis

TABLE 3

The Immed ate Effect of A lrenal ne on the Fpi lermal Cells in the G₂ I hase

	Nr of an I als	Mean per cent of labell d cells	Mean per cent of l l lle l m toses	Mean per cent of unlabelle l ml oses
Controls	4	2 50 + 0 07	0	0 98 ± 0 31
10 micr g adrenal ne	4	2 23 + 0 34	0 50 + 0 06	1 2 ± 0 14

DISCUSSION

Experiment no 1 demonstrates that adrenaline in considerable doses immediately speeds up the mitotic rate Experiment no 2 demonstrates

females Histological sections from the back skin have been stained with haematoxylin-eosin and mitoses have been scored among 2 000 nucleated epidermal cells The colcemid technique has been used to calculate the mitotic duration by using the formula of *Leblond & Stevens* (1948)

Experiment No 1.

The immediate effect of adrenaline on the epidermal mitotic rate of hairless mice Three groups of eight animals each were injected with 0 1 mg of colcemid in 0 25 ml of saline solution Two hours later, when the colcemid was assumed to have arrested a uniform number of mitoses in all of the animals, group no 1 was injected intraperitoneally with 10 microgrammes of adrenaline In group no 2 the "alarm reaction" was provoked by exposing the animals to intense sound of 30 seconds duration every five minutes during half an hour Group no 3 served as control animals All groups were killed three hours after injections of colcemid, i e one hour after the onset of the adrenaline effect in groups no 1 and 2 The results are shown in Table 1 and demonstrate that adrenaline immediately speeds up to epidermal mitotic rate in hairless mice

TABLE 1

The Immediate Effect of Adrenaline and the Alarm Reaction on the Epidermal Mitotic Rate in Colcemid Treated Animals

	Number of animals	Mean per cent of mitoses arrested with colcemid	Mean per cent increase in arrested mitoses during one hour
Controls	8	1 45 ± 0 14	
10 micr g adrenaline	8	2 13 ± 0 12	0 68
Animals in 'alarm reaction	8	1 97 ± 0 15	0 52

Experiment No 2

The prolonged effect of adrenaline on the epidermal mitotic rate of hairless mice Two groups of four mice each were injected with 0 1 mg of colcemid in 0 25 ml saline solution Shortly after the colcemid injection the animals of groups no 1 were injected with 3 microgrammes of adrenaline in 0 03 ml saline solution These animals were given a similar injection of adrenaline two hours later, and after a new period of two hours they were injected with 4 microgrammes of adrenaline in 0 04 ml saline solution During the experimental period the animals received 10 microgrammes of adrenaline in 0 10 ml saline solution

Group no 2 received injections of saline solution without adrenaline parallel to the adrenaline injections of group no 1 and they received 0 1 ml of saline solution during the experimental period In group no 1 the number of mitotic figures was 1 24 ± 0 12 per cent and in group no

2 the number of mitosis was 2 2 ± 0 13 per cent of the nucleated epidermal cells These results demonstrate obviously that 0 1 mg of colcemid does not arrest the epidermal mitosis for five hours in these animals when they are under influence of high doses of adrenaline (see Table 2)

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The stripping film technique was used for autoradiography, the film was exposed to the histological sections for 14 days, and after developing and fixing of the film the histological sections were stained with haemotoxylin throug the film emulsion Labelled cells, labelled mitoses, and unlabelled mitoses were scored among 2 000 nucleated epidermal cells in each animal The results are shown in Table 3 and demonstrate that the immediate effect of adrenaline on the epidermal cells in G_2 phase is to give them an impetus to enter mitosis

TABLE 3

The Immediate Effect of Adrenaline on the Epidermal Cells in the G_2 Phase

	No of animals	Mean per cent of labelled cells	Mean per cent of labelled mitoses	Mean per cent of unlabelled mitoses
Controls	4	2 50 ± 0 07	0	0 98 ± 0 41
10 micr g adrenaline	4	2 23 ± 0 33	0 50 ± 0 06	1 2 ± 0 14

DISCUSSION

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females Histological sections from the back skin have been stained with haematoxylin-eosin and mitoses have been scored among 2 000 nucleated epidermal cells The colcemid technique has been used to calculate the mitotic duration by using the formula of *Leblond & Stevens* (1948)

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Kreyberg L & Evensen A Influence of stress on the diurnal rhythms in mitotic count and in mitotic duration in the epidermis of hairless mice Under preparation
Leblond C P & Stevens C F The constant renewal of the intestinal epithelium in the albino rat Anat Rec *100* 357 371 1948

that colcemid in the concentration used (0 1 mg per mice) is not able to arrest the epidermal mitoses for as long time as five hours under influence of great doses of adrenaline Experiment no 3 demonstrates that the immediate effect of adrenaline on the mitotic rate is to give the cells in the G_2 phase an impetus to enter mitosis The shortest duration of the G_2 phase observed in our hairless mice is 55 minutes (*Evensen* 1962), but usually labelled mitoses are not seen before one hour and *fifteen minutes after the injection of tritiated thymidine In experiment* no 3 forty labelled mitotic figures were found among 2 000 epidermal cells in 4 animals which were injected with adrenaline, whereas no labelled mitoses was observed among the same number of epidermal cells in the animal group which did not receive injection of adrenaline

Evensen & Heldaas (1964) have demonstrated *in vitro* that the colcemid effect on epidermal mitoses is markedly shortened when adrenaline is added to the culture media The results of *Bullough* (1955), *Gelfant* (1960), and *Bullough & Laurence* (1961) may therefore *easily be understood as they prepared their histological sections* after the epidermal cells had been under influence of adrenaline for 4 or 5 hours

It is obvious from experiment no 2 that the duration of the colcemid effect is shortened during influence of adrenaline as the number of arrested mitoses 5 hours after the first adrenaline injection is less than the number of arrested mitoses one hour after injection of adrenaline (Table no 1)

It is impossible to conclude from these experiments if adrenaline speeds up the whole cell proliferation cycle or if the effect is to shorten the G_2 phase only The effect of adrenaline on the rate of DNA synthesis may be studied by repeated injections of tritiated thymidine (*Evensen* 1962)

SUMMARY

Experiments have been performed that demonstrate that adrenaline has not an antimitotic effect On the other hand it has a markedly promoting effect on the epidermal mitotic rate during the first hour *of adrenaline administration The mode of the adrenaline effect* is discussed

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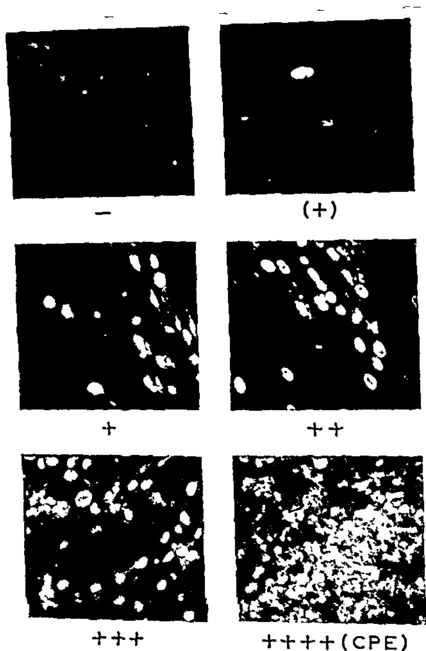


Fig 1.
For explanation see text

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From the State Serum Institute Helsinki Finland

POLYOMA VIRUS AND MOUSE STRAIN SUSCEPTIBILITY

By

MATTI JAHKOLA and TAPANI VAINIO

Received 18 ix 63

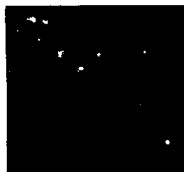
Most inbred mouse strains show an equal susceptibility to the tumour producing effect of polyoma virus grown in tissue culture Thus *Dawe et al* (1959) found only two strains, namely C57Bl/Ka and C57Br/cd with a relative resistance to the oncogenic effect of polyoma virus In their experiments, from five to ten per cent of the animals of these strains developed tumours when inoculated with virus preparations which in some other inbred strains produced tumours in more than 50 per cent of the animals No further studies on these lines have been reported, however Hence we have attempted to pursue this problem still further

MATERIAL AND METHODS

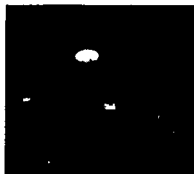
C57Bl/6 and DBA/2 mice as well as their F1 hybrids were used for *in vivo* studies Newborn mice less than 24 hours old were inoculated with 0 05 ml of a single batch of polyoma virus made in a mouse embryo fibroblast culture The haemagglutinating titre of this preparation assessed by the method of *Fogel & Sachs* (1959) was 1 32 The virus was stored at —70° C in glass tubes containing 0 5 ml aliquots and melted at 37° C just prior to inoculation The animals were checked for tumours once weekly and autopsy was performed upon death or when the tumours had grown to a considerable size The observation period was 12 months for C57Bl and F1 mice and 8 months for DBA mice Histological specimens were taken from all visible tumours the parotid submaxillary and sublingual glands and from the kidneys In addition sections of the spine were taken from 13 and 16 mice of C57Bl and DBA strains respectively

For *in vitro* studies primary tissue cultures of 15 to 17 day old whole mouse embryos were used The culture medium consisted of Eagle s basal medium in Earle s balanced salt solution supplemented with 10 per cent inactivated horse serum The cells were grown in Leighton tubes or Petri dishes provided with coverslips When Petri dishes were used the cultures were incubated in 5 per cent CO_2 in air and the medium contained 0 17 per cent of sodium bicarbonate The virus used for *in vitro* studies had an HA titre of 1 512 It was inoculated at the time of plating of cells so that the final dilutions were 10^{-} and 10^{-} The culture medium which was renewed daily was titrated for haemagglutinins Viral cytopathic effect and the number of cells containing viral antigen were recorded daily For the latter procedure the fluorescent *antibody technique was used as described by Vainio (1963)* In numbering the cells containing viral antigen as judged by the fluorescent antibody technique six categories were used and are indicated in Fig 1

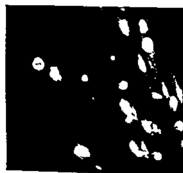
This investigation was supported by a grant from *Sigrid Juselius Foundation*



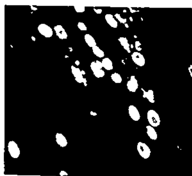
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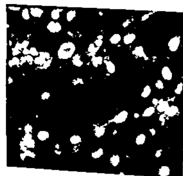
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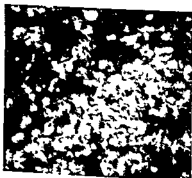
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Fig 1
For explanation see text

RESULTS

In vivo Studies

The results of *in vivo* experiments are summarized in Table 1 Owing to the low potency of the virus preparation used, the percentage of tumours in DBA/2 strain was only 43 per cent There was, however, a significant difference in the number of tumours in DBA/2 and C57Bl6 strains, the latter showing an incidence of 7 per cent F_1 hybrids behaved like their less susceptible parent strain, and it was found to be of no significance whether the mother was of the resistant or susceptible strain The appearance of tumours in DBA/2 strain mainly accumulated in the age period of 3 to 5 months For the other mice no such period could be stated due to the small number of tumours 33 per cent of DBA/2 mice had multiple tumours, many of which could only be discovered histologically In addition to the parotid glands, tumours were found in five different organs, namely the sublingual and submaxillary glands, mammary glands, vertebrae and kidneys The histological features of the tumours were the same as those reported in previous studies (*Law et al* 1955, *Dawe et al* 1959, *Stanton et al* 1959) As for the parotid tumours, they were bilateral in 13 of the 20 tumour-bearing DBA/2 mice (65 per cent), whereas no bilateral tumours were found in the other mouse series No conclusions could be drawn from the length of the period between tumour appearance and death, because most animals were killed after the tumours had either grown to a size of 10 mm in diameter, or if they had ulcerated

In vitro Studies

The *in vitro* studies were performed using embryonic cell cultures of four inbred mouse strains H Albany, AKR/Lwn, DBA/2 and C57Bl6 The results obtained are illustrated in Fig 2 The first signs of viral effect were observed on the 3rd or 4th day in all cultures in which the 10^{-2} virus concentration was used In the cultures infected with 10 dilution of the virus preparation the viral effect could be recorded after additional 3 days In all the mouse strains the three different parameters of viral growth, *e g* cytopathic effect, the production of viral haemagglutinin and the relative number of the cells containing viral antigen were of the same order No difference in the amount of virus, assessed by haemagglutination, could be detected between the different mouse strains

DISCUSSION

The incidence of polyoma tumours in C57Bl6 and DBA/2 mouse strains reported in this study is in accordance with the results obtained by *Dawe et al* (1959) if the low potency of the virus preparation used is taken into consideration The low incidence of tumours in F_1 hybrids would suggest that the resistance to polyoma oncogenesis displayed by

TABLE 1

Age in months	DBA 2				DBA ♂ × C57 BL ♀				C57BL ♂ × DBA ♀				C57 BL 6			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
3	46	3	7	3	12		–		14	–	–	–	29	1	–	–
4	43	8	6	4	12	–	–	–	14	–	–	–	29	–	–	
5	35	6	4	1	12	1	–	–	14	–	–	–	28	1	–	–
6	31	3	2	–	12	–	–	–	14	–	–	–	27	–	–	–
8	26	–	–	–	12		–	–	14	–	–	–	27	–	–	–
12					10	–	–	–	13	–	–	1	24	–	–	(1)§
Total	46	20	15	8	12	1	–	–	14	–	–	1	29	2	–	(1)

1 — Animals alive
2 — With parotid tumours
3 — With multiple tumours
4 — With other than parotid tumours
§ — myoepithelioma of the neck

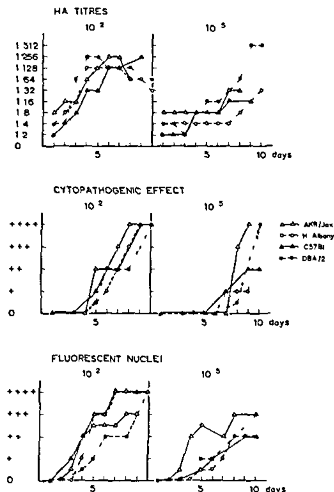


Fig 2
For explanation see text

C57Bl/6 strain would depend on one or several hereditary factors, which dominate the factors for tumour susceptibility A similar kind of genetic resistance has been observed in the C3H mouse strain against murine hepatitis virus (*Bang & Warwick 1960*) and in the *PRI* mouse *strain* against Arbor B viruses (*Sabin* 1952) The mechanism of the resistance to Arbor B viruses has been further studied by *Theis & Koprowski* (1962) and *Vainio* (1963) These studies indicate that in genetically resistant mice most tissues support the replication of Arbor B viruses In this respect the splenic macrophages make an exception so that cells originating from a resistant strain appear incapable of supporting the replication of the named viruses This would imply that the susceptibility or resistance to a given virus and to the disease caused by it need only be reflected in the behaviour of a few specialized cells of the organism This would explain the susceptibility of C57Bl 6 embryonic fibroblasts to polyoma virus reported in this study and elsewhere (*Winocour & Sachs* 1960)

The oncogenic effect of polyoma virus is closely related to the inocu-

lation time, in that only mice inoculated as newborns develop tumours The virus must then attack its target organs when these are at a particular stage of development This stage might be reached earlier in a resistant strain, a suggestion which is supported by the studies of *Auerbach* (1961) on differences in the rate of lymphatic leukaemia in AKR and C3H strains of mice He demonstrated that at birth the thymus of AKR mice was as differentiated as the thymus of C3H mice at an age of two days The former strain has a high rate of lymphatic leukaemia It is of interest in this context that the postnatal increase of H 2 antigen in C57Bl mouse spleen and liver cells reaches the adult level several days earlier than in the same cells of C3H and A mice (*Moller* 1963)

The immunological response elicited by polyoma virus in the two mouse strains might also be thought of as contributing to the differences in tumour incidence In our studies so far (*Jahkola* 1963) no difference has however, been found in the haemagglutination inhibition titres of the two mouse strains inoculated as newborns with polyoma virus

After the relative resistance of F_1 hybrids to polyoma virus oncogenesis was discovered other series of experiments were started using a more potent virus preparation, and in addition to the parent strains F_1 and F_2 generations were included This work is in progress

SUMMARY

Polyoma virus infection has been studied in C57Bl 6 and some other mouse strains to elucidate the relative resistance of the former mouse strain to polyoma virus oncogenesis *In in vivo* experiments using a virus preparation of moderate potency an incidence of 43 per cent of tumours could be recorded in DBA/2 mice inoculated with virus as newborns whereas the incidence was under 10 per cent in C57Bl 6 mice and F_1 hybrids treated in the same way Multiple tumours were only observed in the DBA 2 strain *In in vitro* studies no differences could be established in the course of polyoma infection in embryonic fibroblast cultures of the C57Bl 6 and four other mouse strains

The different possibilities for the mechanism of hereditary resistance to viral oncogenesis are discussed

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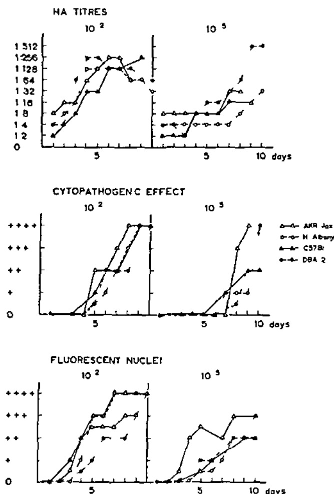


Fig 2
For explanation see text

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Kapte n W Wilhelmsen og Frues Bakter log ske Institutt Oslo U n versity an l
R kshospitalet Oslo (Hea l S D ck Henrik en M D)

ACUTE EXPERIMENTAL TOXOPLASMOSIS IN MICE TREATED WITH SOME NEW CHEMOTHERAPEUTICS AND ANTIBIOTICS

By

TORE MIDTVEDT

Received 11 x 63

During the years since the discovery by *Wolf & Cowen* (1937) that Toxoplasma gondii is the cause of human disease numerous reports have appeared describing studies of the chemotherapy in experimental toxoplasmosis

Toxoplasma gondii behaves as an obligate intracellular parasite and as far as known it has not been cultivated in media without living cells As pointed out by *Steen* (1950) the effect of chemotherapy on Toxoplasma can therefore only be studied either by giving the drug to animals infected with Toxoplasma or by incubating toxoplasma containing materials with the drug and then testing the viability of the protozoa by injecting the mixture into living organism

The earliest work on the chemotherapy of toxoplasmosis was reported by *Sabin & Warren* (1941) They found that sulphonamide [illegible]

[illegible] greatly varying degrees of activity have been found A brief survey of these problems is given by *Eyle* (1956)

Another chemotherapeutic agent with some effect in experimental toxoplasmosis is pyrimethamine (*Eyle & Coleman* 1952 *Summer* 1953) A synergistic effect of pyrimethamine and sulphonamides in experimental toxoplasmosis has also been reported (*Eyle & Coleman* 1953 *Eyle & Coleman* 1955 b *Eyle & Jones* 1955) Scandinavian workers have described good effect in human chorioretinitis presumed to be due to Toxoplasma with atebrin and with atel [illegible]

[illegible] tested against experimental toxoplasmosis The most active compound yet tested probably is chlortetra

The author is indepted to I *Torgersen* for excellent techn cal as stance

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1-ethyl 7 methyl 1 8 napthyrid n 4 1 3 carboxylic acid (Win 18320) is a new chemotherapeuticum synthesized at the Sterling Winthrop Research Institute New York It seems to have an excellent effect against Gram negative organisms com [illegible] as is known the drug has n t

is one of the so called long ensively tested against micr toxoplasmosis (Domagk et al rkusen

The drugs were mixed daily in the food and it was controlled that nearly all the food was eaten The daily d age of each drug given was as recommended in the previously ment ned literature on the treatment of experimental infection other than toxoplasmosis in mice The food was commercial pellets obtained from P [illegible] lars n & Co Oslo

RESULTS

The first problem which was attacked was the therapeutic activity of the drugs mentioned before In Table 1 some experiments have been recorded The drug therapy was started in all series 4 hours before the inoculation with the diluted exudate Each drug was also given to 10 uninfected mice in the same dosage as in the experiments All these mice remained well for at least 30 days

From the data presented in Table 1 it will be seen that there was no effect of Ledermycin Doktacillin Flagyl and Win 18320 There are no survivors after 30 days and the duration of the disease is as in the untreated group The results indicate that further experiments with these four substances are unnecessary

TABLE 1

The Effect of some new Drugs on Experimental Toxoplasmosis in White Mice

Group	Mice	Daily treatment for 20 days	Day of death*	Survivors after 30 days
A	15	Aureomycin ? mg	8 8 8 9 9 10 10 15 2? 23 25	5
B	15	Ledermycin ? mg	7 7 7 7 7 7 7 7 7 7 8 8 8 8 8	None
C	15	Doktacillin 3 mg	7 7 7 7 7 7 7 8 8 8 8 8 8 8 8	None
D	15	Flagyl 2 mg	6 6 7 7 7 7 7 7 7 7 7 8 8 8 8	None
E	15	Win18320 0 8 mg	7 7 7 7 7 7 7 7 8 8 8 8 8 8 8	None
F	15	Bayrena 2 mg	No one died	15
G	40	No treatment	6 6 6 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8	None

* Counted from the day of infection this day not included

cycline (*Summers* 1949, *Steen* 1950, *Kåss & Steen* 1951) Terramycin has no or only slight effect (*Grassi & Kåss* 1952, *Gronroos* 1953) Penicillin, streptomycin, chloramphenicol, erythromycin, puromycin, bacitracin, neomycin, polymyxin and fumagillin have also been tested and many of them have been reported to possess some activity (Survey by *Eyle* 1960) But as pointed out by Eyle, none of the antibiotics yet tested seemed to be sufficiently active to be of practical use in the treatment of human toxoplasmosis But he also pointed out that the fact that some activity against experimental toxoplasmotis is found in many antibiotics makes it essential to study new antibiotics as they become available

On these premises we felt it would we of interest to see whether some of the new drugs were effective against experimental toxoplasmosis in white mice The drugs chosen were Ledermycin, Doctacillin, Flagyl, Win 18320 and Bayrena As far is known, none of these have as yet been tested against experimental toxoplasmosis

Following *Steen* (1950), the experiments were planned in order to elucidate the following problems

1 Is the drug therapeutically active?
2 If effective, how late may the treatment be instituted?
3 Does the therapy eradicate the parasites, or do the cured mice remain carriers?
4 Are the cured mice immune against a new infection with Toxoplasma gondii?

MATERIALS AND METHODS

Toxoplasma Strain

The 'RH' strain of Toxoplasma gondii first isolated by *Sabin* (1941) was used in all the experiments

Mice

Young male white mice weight about 20 g were used When mice are infected with Toxoplasma by the intraperitoneal route there is an abundant peritoneal exudation during the terminal stage of infection In our experiment the exudate was taken on the fourth day after the inoculation Penicillin (ca 1000 I E per ml) and streptomycin (ca 100 mcg per ml) were added and the exudate was diluted 1 to 1000 in sterile saline This diluted peritoneal exudate was injected intraperitoneally and the dose was 0 5 ml per mouse in all the experiments

Chemotherapeutics and Antibiotics

As an antibiotic with a previously known effect on experimental toxoplasmosis ' – The drug was kindly supplied by Lederle was also supplied by Lederle The drug [illegible] ated to chlortetracycline (*Finland* 1960) acid (Doktacillin) was kindly supplied by Norsk Astra A S Oslo [illegible] cillin derivative originally produced by Beecham Research Laboratories England and with an antimicrobial spectrum somewhat similar to that of the broad spectrum antibiotics *e g* chlortetracycline (*Brown & Acred* 1961, *Rolinson & Stevens* 1961 *Stewart Coles & Nixon* 1961)

1 β hydroxyethyl 2 methyl 5 nitro imidazol (Flagyl) is a new aminitrozol derivative with good effect against infections with the protozoon Trichomonas vaginalis (*Cosair & Julou* 1959) Flagyl was kindly supplied by May and Baker Ltd

1 ethyl 7 methyl 1 8 napthyridin 4 1 3 carboxylic acid (Win 18320) is a new chem therapeuticum synthesized at the Sterling Winthrop Research Institute New York It seems to have an excellent effect against Gram negative organisms commonly path genic to man (Lesher *et al* 1962) As far as is known the drug has not

aytena) is one of the so called long ery intensively tested against micro mental toxoplasmosis (*Domagk et al* er Leverkusen

The drugs were mixed daily in the food and it was controlled that nearly all the fo d was eaten The daily dosage of each drug given was as recommended in the previously menti ned literature on the treatment of experimental infecti n other than toxoplasmosis in mice The f od was commercial pellets obtained fr m P Larsen & Co Oslo

RESULTS

The first problem which was attacked was the therapeutic activity of the drugs mentioned before In Table 1 som experiments have been recorded The drug therapy was started in all series 4 hours before the inoculation with the diluted exudate Each drug was also given to 10 uninfected mice in the same dosage as in the experiments All these mice remained well for at least 30 days

From the data presented in Table 1 it will be seen that there was no effect of Ledermycin Doktacillin Flagyl and Win 18320 There are no survivors after 30 days and the duration of the disease is as in the untreated group The results indicate that further experiments with these four substances are unnecessary

TABLE 1

The Effect of some new Drugs on Experimental Toxoplasmosis in White Mice

Group	Mice	Daily treatment for 20 days	Day of death*	Survivors after 30 days
A	15	Aureomycin 2 mg	8 8 8 9 9 10 10 15 2[illegible] 23 25	5
B	15	Ledermycin 2 mg	7 7 7 7 7 7 7 7 7 7 8 8 8 8 8	None
C	15	Doktacillin 3 mg	7 7 7 7 7 7 7 8 8 8 8 8 8 8 8	None
D	15	Flagyl 2 mg	6 6 7 7 7 7 7 7 7 7 7 8 8 8 8	None
E	15	Win18320 0 8 mg	7 7 7 7 7 7 7 7 8 8 8 8 8 8 8	None
F	15	Bayrena 2 mg	No one died	1[illegible]
G	40	No treatment	6 6 6 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8	None

* Counted from the day of infection this day not included

TABLE 2

Variations in the Treatment of Experimental Toxoplasmosis with Bayrena

Group	Mice	Daily treatment	Duration	Day of death	Survivors after 30 days
I	15	2 mg	20 days	No one died	15
II	15	2 mg	30 days	No one died	15*
III	15	2 mg	8 days	20, 23 23, 24 26, 29	4§
IV	15	2 mg	3-23th day after infect	9	14
V	15	1 mg	20 days	12, 18 19	12
VI	15	0 5 mg	20 days	9 9 9, 11, 12, 18 18 18 22	6
VII	15	No treatment		6 7, 7 7 7 7 7, 7, 7, 7, 7, 7, 7, 7, 7 7, 8 8 8 8	None

* Survivors after 40 days

§ 5 mice killed on the 10th day after the end of the treatment when testing the carrier state of the survivors

Table 1 also demonstrates a certain effect of Aureomycin One third of the treated mice survived after 30 days and there is also an increase of the survival time of the non-survivors

The most effective drug tested in our experiments was Bayrena As shown in Table 1, none of the treated mice died within 30 days and, in contrast to the mice treated with Aureomycin, none of the survivors showed symptoms of infection

In a following series of experiments, the effect of variations in dosage and duration of the treatment with Bayrena was studied In all experiments except one, the treatment was started 4 hours before the inoculation In the last one, the treatment was instituted on the third day after inoculation in order to elucidate the problem of how late the therapy may be started to show effect The results have been recorded in Table 2 As shown in the table, an effect is obtained by 2 mg of Bayrena daily even when the period of therapy is as short as 8 days Also when the therapy was instituted on the third day after inoculation, the effect of Bayrena was good The effect of treatment after longer duration of the drug-free period was not studied since many of the infected mice became clinically ill on the fourth day after inoculation and therefore did not eat as much as before A peroral therapy would therefore vary too much to be of interest

When the daily dose of Bayrena was reduced to 1 mg for 20 days, 3 mice died The time of death was delayed as compared with the control group, and some of the survivors became ill during the second week after inoculation With 0 5 mg of Bayrena daily for 20 days the

number of survivors decreased by one half, and almost all of them showed signs of illness during the second week after the inoculation

In the next series of experiments *the cured mice were tested for* carrier state Approximately one third of the surviving mice from each group was used The mice were killed on the 10th day after the end of the treatment Saline extracts of the brains from each of these mice were injected separately intraperitoneally into 4 other mice The results are shown in Table 3 All these new mice except 4 from each of the 3 groups treated with 2 mg of Bayrena for 20 or more days died within 13 days and terminally they had an abundant peritoneal exudate containing many toxoplasma parasites After 30 days 3 of the 12 surviving mice were inoculated with diluted toxoplasma containing exudate and these mice *died on the 8th day after inoculation*

TABLE 3

Carrier State Tested on the 10th Day after the End of the Treatment

Group	No of mice tested	No of mice inoculated	Day of death	Survivors after 30 days
A	2	8	7 7 7 7 8 8 8 8	None
I	5	20	7 7 8 8 8 8 8 8 8 8 8 8 9 9 9 9	4
II	5	20	8 8 9 9 9 9 9 10 10 10 10 10 10 10 10 13	4
III	5	20	5 5 5 5 6 6 7 7 8 8 8 8 9 9 9 9 9 9 9 9	None
IV	5	20	7 7 7 8 8 8 8 8 9 9 9 9 9 10 11 13	4*
V	4	16	3 8 10 10 10 10 10 10 10 10 10 10 11 11 11 11	None
VI	2	8	6 7 7 7 7 8 8 9	None

* The 4 survivors were infected with the same brain

Approximately one third of the remaining mice from the two first series of experiments except group III (Table 2) was used in a new series of experiments to test whether the cured mice were *immune* against a new intraperitoneal infection with toxoplasma The last one third of the cured mice was used as a control group The new inoculations were made on the 10th day after the end of the treatment As shown in Table 4 only 4 of the previously infected mice died after reinoculation All these four mice were from groups *treated for 20* days or longer

TABLE 4

Effect of Reinoculation with Toxoplasma gondii *in Cured Mice on the 10th Day after the End of the Treatment*

Group	Survival rates of the mice		Day of death of non-survivors
	Reinoculated	Not reinoculated	
A	2 / 2		—
		1 / 1	—
I	4 / 5		7
		5 / 5	—
II	2 / 5		8 8 9
		5 / 5	—
IV	5 / 5		—
		4 / 4	—
V	4 / 4		—
		3 /4	7
VI	2 /2		—
		2 /2	—
VI *Controls**		*0 / 10*	7 7 7 7 7 *8 8 8 7 7*

* Normal previously uninoculated mice

DISCUSSION

When white mice are inoculated with *Toxoplasma gondii* by the intraperitoneal route, the infection is invariably fatal to untreated mice. Previously it has been found that Aureomycin has some effect on experimental toxoplasmosis in white mice, and our results confirm this. As also found by others (*Steen* 1950), we find that the treatment cannot eradicate the paratites. The treated mice remain carriers, and they are immune against a new infection with toxoplasma.

Grassi & Kåss (1952) pointed out that Terramycin, in contrast to Aureomycin, has no effect against experimental toxoplasmosis in mice. Our results seem to indicate that Ledermycin—demethylchlortetracycline—behaves as Terramycin. The effect of tetracyclines may perhaps vary in different animals. *Girous & Gaillard* (1951) are of the opinion that Terramycin *is more active than Aureomycin in the treatment* of experimental toxoplasmosis in rabbits.

The effect of some new drugs, Doktacillin, *Flagyl and Win 18320, in* the treatment of experimental toxoplasmosis in mice was tested and we found no effect.

When using a derivative of sulphamethoxypyridazin—Bayrena—there was a marked effect. After dosages of 2 mg daily for 20 days or longer all the mice survived. In some few cases in these groups the survivors did not remained carriers, the therapy had eradicated the para-

sites In the same groups some of the mice died after reinoculation they had not developed resistance against Toxoplasma gondii

When the dosage of Bayrena was reduced to 1mg or 0 5 mg daily all the survivors tested seemed to be carriers and they were also immune against reinoculation

As mentioned before greatly varying degrees of activity have been reported when testing the antitoxoplasmatic activity of sulphonamides According to *Fyle* (1960) the most effective sulphonamides yet tested seemed to be sulphamethazin sulphapyrazin sulphamerazin and sulphadiazin A daily dosage of sulphapyrazin of approximately 70 mg per kg gave 100 per cent survivors and some of them were non carriers (*Eyle & Coleman* 1955 a) These experiments were made with a local strain of Toxoplasma gondii In other experiments (*Fyle & Coleman* 1955 b) it was found that the effect of the treatment against an infection with the RH strain in mice was similar to that found with the local strain

In our experiments 100 per cent survivors were found when the treatment was 2 mg daily or approximately 100 mg per kg This dosage is similar to that found for the best sulphonamides so far tested

Previously it has been pointed out that the treatment with sulphonamides frequently is followed by relapse and death of the animals after the discontinuation of the drug (*Eyle* 1960) In our experiments all except one of the survivors tested survived for at least 30 days after the end of the treatment

Thus our experiments may be taken to indicate at least as good effect of Bayrena as of the most effective sulphonamides yet tested even if too definite conclusions would be premature at the present stage

SUMMARY

The effect of some new drugs on experimental toxoplasmosis in white mice was tested

Ledermycin Doktacillin Flagyl and Win 18320 were found to have no effect

A derivative of sulphamethoxypyridazin—Bayrena— was found to be as effective in the treatment as the best sulphonamides yet tested

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From the Enterovirus Department Statens Seruminstitut C penhagen Denmark

CONVENTIONAL TISSUE CULTURE NEUTRALIZATION TECHNIQUE EMPLOYED IN SERODIFFERENTIATION BETWEEN TYPE 1 POLIOVIRUSES

By

FIN HAHNEMANN and KNUD SIBONI

Received 2 x 63

Different kinds of neutralization tests have been used for intratypic serological differentiation of poliovirus strains *McBride* (1) found none of the conventional end point methods sufficiently sensitive to characterize uniquely a given poliovirus strain and he employed in his studies a kinetic method measuring the neutralization rate *Wecker* (2) used plaque neutralization tests with antiserum incorporated in the agar overlay and several authors (3 12) have used one or both of these methods or modifications of them with good results *Wenner* (10), however was able to differentiate a considerable number of poliovirus strains by means of conventional neutralization tests and so was *Gard* (14) using immuno-inactivation

The immunization procedures used by different authors for production of antisera sufficiently specific for serodifferentiation have been subject to great variation but the only factor which seems to have influenced the specificity of the sera has been the length of the immunization period Several authors (1 8, 14 15) have found a tendency to higher specificity of the sera after a short than after a long period of immunization

In this laboratory plaque neutralization tests have been employed in a previous study (15) In the present work, the specificity of rabbit antisera produced by rapid immunization with 3 virulent and 3 attenuated strains of polio virus type 1 was studied employing conventional neutralization tests in tissue culture roller tubes

MATERIALS AND METHODS

Tissue cultures Trypsinized monk 1 ... rom Cynomolgus ... Hanks BSS with ... hey were used on ... 199 without any

Virus strains Three virulent and three attenuated strains of polio virus type 1 were used

1 Mahoney
2 7920 I isolated 1961 from the stools of a 9 year old boy with paralytic polio
3 10133 I isolated 1962 from faecal material from a 14 year old boy with paralytic polio
4 LSc 2ab Sabin's attenuated type 1 strain
5 10077 II isolated 1962 from a 6 year old healthy boy 4 days after feeding of Sabin type 1 vaccine (LSc 2ab) manufactured by the Connaught Laboratories Canada
6 10077 VIII isolated 1962 from the *above mentioned healthy boy 43 days after* the vaccination

The Mahoney and LSc 2ab strains were kindly supplied by Dr Albert B Sabin and have been used after 2 monkey kidney tissue culture passages in our laboratory

Stock viruses For the production of stock virus material the virulent strains were grown at 37° C the attenuated strains at 34° C The tissue culture fluids were harvested after complete degeneration of the cells and stored in 1 ml amounts at —20° C

Virus titrations The titrations were performed using ten fold dilutions and 5 roller tube cultures per dilution An inoculum of 0.2 ml was employed and the —$\log_{10}$ titre was calculated ad modum Kärber and expressed per 0.2 ml All virus stocks were titrated repeatedly at 37° C before use in the experiments and both virus titrations and virus controls were included in each experiment

Immune sera Groups of six rabbits weighing 3000 3850 g were inoculated with one of the undiluted virus strains without any adjuvans in the following way 7 ml subcutaneously 1 ml intradermally and 2 ml intravenously on the same day Bleeding by heart puncture was done after one week The sera were inactivated at 56° C for 30 min and stored in 1ml amounts at —20° C

Serum titrations were performed in the conventional way employing serial 2 fold serum dilutions in saline and a constant amount of virus approximately 100 TD_{50} in the final inoculum The serum virus mixtures were incubated for 1 hour at 37° C in a water bath and afterwards stored in a refrigerator at 4° C until inoculation Two roller tube cultures were used per serum dilution and the cultures were incubated at 37° C During the first two days they were rotated in a roller drum but subsequently they were left stationary Readings were made on the 4 and 7 day and the last reading was used for calculation of the titres by the method of Kärber All serum titres are in this study recorded as —$\log_2$ values

EXPERIMENTAL

The titres from the cross neutralization tests on the 36 rabbit sera against the 6 virus strains are listed in Table 1 They were obtained in one experiment *performed in the course of one week* The serum dilutions were stored in a refrigerator at 4° C and on different days titrated against 2 virus strains one virulent and one attenuated at a time The standard deviation in a single neutralization test in roller tubes S_N has been found to be 0.6 log (15) Therefore the standard deviation on the difference between two titres S_D is $\sqrt{S_N^2 + S_N^2} = S_N \sqrt{2} = 0.85$ log In this experiment the average of 3 titrations was used for the calculations Consequently the S_D was reduced by the factor $\sqrt{3}$ to 0.5 log and a difference of 1.0 log was significant (P = 0.05)

As it may be seen from Table 1 the serological difference between the virulent and the attenuated groups of virus strains was considerably greater than the mutual differences between the single virulent or attenuated strains respectively However not all the sera were specific The group specificity of *an individual serum has been calculated by*

TABLE 1

typic Serodifferentiation Test with 36 Rabbit Antisera Prepared against 3 Virulent and 3 Attenuated Polio Type 1 Strains Cross Neutralization Tests in Roller Tubes

sera	Serum no	Virus strains								Difference*
		Virulent				Attenuated				
		Mahoney	990 I	10133 I	Mean	LSc	II	VIII	Mean	
oney	601	12.0§	12.5	13.0	12.5	11.5	10.0	10.0	10.5	+2.0
	602	9.0	9.0	9.5	9.2	9.0	8.0	9.0	8.7	+0.5
	603	8.5	8.0	9.5	8.7	9.0	8.0	10.5	9.2	—0.5
	604	12.0	12.0	11.5	11.8	11.0	9.0	10.5	10.2	+1.6
	605	9.0	9.5	10.0	9.5	8.5	8.5	9.0	8.7	+0.8
	640	11.0	11.0	9.5	10.5	9.5	8.5	9.0	9.0	+1.5
0 I	631	10.0	10.5	10.5	10.3	11.5	10.0	9.5	10.3	0.0
	632	12.0	12.5	13.0	12.5	13.0	12.5	12.0	12.5	0.0
	633	10.0	10.5	9.5	10.0	9.5	9.0	9.5	9.3	+0.7
	634	9.5	9.5	10.5	9.8	10.0	8.5	9.0	9.2	+0.6
	635	11.0	12.0	11.0	11.3	10.5	10.5	11.5	10.8	+0.5
	636	10.0	10.0	10.0	10.0	10.0	9.0	9.0	9.3	+0.7
33 I	606	8.5	10.0	10.5	9.7	10.0	8.0	10.0	9.3	+0.4
	611	10.0	10.0	10.5	10.2	10.5	10.0	9.5	10.0	+0.2
	617	9.0	10.0	10.0	9.7	8.0	8.0	9.0	8.3	+1.4
	624	9.0	10.0	9.0	9.3	9.5	8.0	10.5	9.3	0.0
	629	9.0	9.5	9.0	9.2	8.5	7.5	8.0	8.0	+1.2
	638	9.0	8.5	9.0	8.8	8.5	9.0	9.5	9.0	—0.2
c	619	11.0	11.0	11.5	11.2	12.0	12.0	12.5	12.2	—1.0
	620	10.0	11.5	10.0	10.5	12.0	12.0	12.0	12.0	—1.5
	621	9.0	9.5	9.5	9.3	11.0	10.5	11.0	10.8	—1.5
	622	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	0.0
	623	9.0	10.0	9.0	9.3	12.0	12.0	11.0	11.7	—2.4
	642	8.5	8.5	8.5	8.5	9.5	9.5	9.5	9.5	—1.0
077 II	607	9.0	10.0	8.5	9.2	9.5	9.5	10.0	9.7	—0.5
	608	10.0	10.0	10.0	10.0	13.0	13.0	11.0	12.3	—2.3
	609	9.5	9.0	9.0	9.2	11.0	12.0	11.5	11.5	—2.3
	610	10.0	10.0	9.5	9.8	13.0	12.5	12.0	12.3	—2.5
	612	10.5	10.5	10.5	10.5	12.5	12.5	11.5	12.2	—1.7
	637	10.5	9.5	10.0	10.0	11.0	12.5	9.5	11.0	—1.0
0077 VIII	625	9.5	9.5	10.0	9.7	11.0	10.5	12.0	11.2	—1.5
	626	8.5	8.0	8.5	8.3	11.0	10.0	11.5	10.8	—2.5
	627	9.0	9.0	9.5	9.2	11.0	10.5	11.5	11.0	—1.8
	628	8.0	8.5	7.5	8.0	12.0	11.0	12.0	11.7	—3.7
	630	8.5	9.0	9.0	8.8	10.5	11.0	11.5	11.0	—2.2
	641	9.5	9.0	8.5	9.0	10.5	10.5	11.5	10.8	—1.8

* Difference between mean serum titre against the 3 virulent virus strains and mean serum titre against the 3 attenuated strains

§ Serum titre —1 g_2 values

taking the difference between the mean serum titres against the 3 virulent strains and the mean serum titres against the 3 attenuated strains As shown in Table 1, five out of 18 antisera (Nos 601, 604, 640, 617 and 629) against the virulent strains and 16 18 of the sera (all but Nos 622

and 607) against the attenuated strains exhibited a significant degree of group specificity

By selection of 3 of the most specific "virulent" of Mahoney antisera and 3 of the most specific "attenuated" of LSc like antisera the differentiation between the Mahoney like and the LSc like groups of virus strains could be greatly improved Employing the formula mean titre of the 3 Mahoney antisera minus the mean titre of the 3 attenuated sera the serological difference between the groups of virus strains became expressed in figures which were *positive for the virulent* and negative for the attenuated strains This difference will be referred to as the Mah/LSc quotient The results are listed in Table 2

TABLE 2

Intratypic Serodifferentiation between 3 Virulent and 3 Attenuated Strains of Polio Virus Type 1 by Cross Neutralization Tests with the Corresponding Antisera

Virus	Difference between mean titre of 18 virulent antisera and of 18 attenuated antisera	Mah/LSc quotient (6 selected antisera*)
Virulent		
Mahoney	+0.6§	+2.7§
7J20 I	+0.8	+2.3
10133 I	+1.1	+2.7
Mean	+0.8	+2.6
Attenuated		
LSc	−1.2	−1.7
10077 II	2.1	−2.7
10077 VIII	1.3	−1.8
Mean	1.5	2.1

* Nos 601 604 640 and 623 610 and 628

§ The calculation is based upon the $\log$ titres of the sera as listed in Table 1

In serodifferentiation tests on Mahoney and LSc 2ab strains in ten different experiments the dose of virus used varied from 125 to 3000 and from 40 to 800 ID_{50} respectively Within these intervals no relationship was found between the dose of virus employed and the Mah LSc quotient

DISCUSSION

The present study has shown the feasibility of using conventional roller tube neutralization tests for demonstration of intratypic differences amongst strains of polio type 1 virus It has been shown that when selected specific antisera are employed the sensitivity and accuracy of this test is equal to other methods employed such as measuring neutralization rate or plaque inhibition For study of strains from patients during mass vaccination campaigns the present technic has the advantage of being very simple and it can thus be handled routinely by technical personnel

The antisera used in the present experiments were produced by giving

rabbits one large dose of live polio virus and bleeding the animals one week later. Inoculation of the attenuated LSc virus gave very good results in that 16 out of 18 rabbits produced sera of good titre and a high degree of specificity. Using the virulent type 1 strains only 5 out of 18 animals responded with the production of antisera which were satisfactory for serodifferentiation tests. This difference cannot be ascribed to differences in titre of the virus used for inoculation of the animals, which was 7.3, 7.0, 6.4 for the 3 virulent strains and 6.9, 7.1, 7.3 for the 3 attenuated strains.

The two type 1 strains, 7920 and 10133, isolated from paralytic patients in this country in 1961 and 1962, respectively, were found to be antigenically closely related to the Mahoney strain, whereas the two strains isolated from a healthy child (No. 10077) 4 and 43 days after he had been vaccinated with LSc, 2ab vaccine were clearly related to the LSc 2ab strain. The 4 strains isolated from human beings were thus not found to be 'intermediate' in the serodifferentiation test. This finding has been confirmed in another study comprising approximately 50 type 1 polio virus isolates (16).

SUMMARY

A total of 36 rabbits were immunized with one large virus dose, either virulent or attenuated type 1 polio virus, and bled after one week. From several of the animals antiserum was obtained which could be used in intratypic serodifferentiation tests, employing conventional neutralization tests in roller tubes. Since the method in its present form involves a considerable number of serum titrations it is hardly cheaper than techniques described previously by other authors; it has, however, the advantage of being very accurate and technically simpler to perform.

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ON THE EFFECTS OF HIGH HYDROSTATIC PRESSURE ON BACTERIA AND BACTERIOPHAGE

1 *Action on the Reproducibility of Bacteria and their Ability to Support Growth of Bacteriophage T2*

By

LARS RUTBERG

Received 11 x 63

Our knowledge of the effects of high hydrostatic pressure on biological material is limited Earlier investigators have found that bacteria are in general unable to grow even at very moderate pressures (50–200 atm) (*ZoBell & Johnson* 1949, *ZoBell & Morita* 1957, *ZoBell & Oppenheimer* 1950) An exception from this rule is illustrated by some bacteria, isolated from ocean depths, that are capable of growth at 600 atm In a series of papers, *Johnson & Lewin* studied the effect of pressure on inactivation of *Escherichia coli* with heat and quinine They found that pressure retarded inactivation at temperatures above those required for optimal growth The effect of pressure on the synthesis of bacteriophage has been investigated in one case (*Foster & Johnson* 1950 51) It was found that pressure increased the latent period and diminished the burst size A summary of the effects of pressure on biological material is found in "The Kinetic Basis of Molecular Biology" (*Johnson, Eyring & Polissar* 1954)

In the present paper experiments are presented which describe the effects of ...

... quent paper

MATERIALS AND METHODS

The bacteria used in this investigation was *Escherichia coli* strain B The phage used was T2 wild type The bacteria were grown in Difco nutrient broth with 5 grams NaCl added per litre The bacteria were assayed for viability by spreading dilutions on L medium (Difco tryptone 10 grams Difco yeast extract 5 grams NaCl

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My sincere thanks are due to Miss *Kerstin Nilsson* for expert technical assistance throughout the experiments

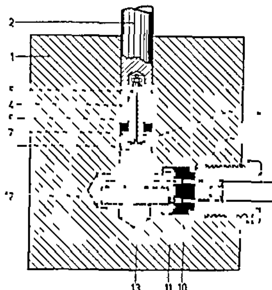


Fig 1

Pressure chamber 1 Steel chamber 2 Main body of plunger 3 Chamber end of plunger 4 Air valve with gasket 5 Valve spring inside threaded plug used for fitting extractor tool 6 Iron washer 7 Teflon gasket 8 Screw nut 9 Gasket adapter 10 Nylon gasket 11 Brass fitting for electrode wire 12 Holder for electrode wire

5 grams, glucose 1 gram Difco agar 12 grams and distilled water 1000 ml) In some experiments adsorbtion of the phage took place in a phosphate buffer pH = 7.0 with 10 grams NaCl and 5×10^{-3} molar $MgSO_4$ added per litre The experiments involving bacteriophage were done according to *Adams* The pressure chamber is shown in Fig 1 Pressure was determined either indirectly from the readings on a manometer giving the oil pressure acting on a hydraulic plunger or directly inside the chamber with the aid of a silver manganin gauge (*Linde* 1939) The results agreed well with each other and in later experiments the manometer was used exclusively The samples were contained in small 2 cc glass vials and sealed with rubber membranes or sterile silicon oil In later experiments the silicon oil was preferred for several reasons It is easier to work with it excludes possible effects of air on the material and it gives less variation between individual experiments Repeated tests have failed to show any external contamination of the vials during experimentation

EXPERIMENTAL

The Inactivation of Bacteria with Pressure

The bacteria were grown aerobically in 20 ml broth in 250 ml flasks on a shaker without any extra aeration to a density of about 5×10 cells/ml They were concentrated 4-5 times in chilled nutrient broth The samples to be tested were put into the glass vials and sealed The samples were then equilibrated to 37° C and exposed to pressure for various times After exposure for the time selected, pressure was returned to atmospheric and the samples assayed for surviving bacteria The rate of application and release of the pressure has been varied from ca 5 seconds to 5 minutes This has not been found to influence the results Survival curves for the bacteria exposed to a number of different pressures are shown in Fig 2 Each point is the average of 5

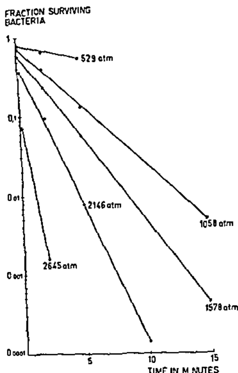


Fig 2

Survival of bacteria exposed to various pressures for various times

independent experiments The curves are exponential, the rate of inactivation increasing with increasing pressure The curves however, extrapolate to points well below 1 This is not related to the time of exposure but only to the amount of pressure applied It is therefore considered to be an effect of the release and/or the application of the pressure Total counts did not change during treatment The experiments on capacity also show that the cells did not lyse during treatment

The Effect of Pressure on the Capacity of the Bacteria

Capacity is defined as the ability of one infected bacterium to produce phage of the same kind used for infection The bacteria were prepared and treated the same way as in the above experiments Immediately after release of pressure viable counts were performed The bacteria were then infected with bacteriophage at a low multiplicity of infection (moi) *e g* less than 0 05 calculated on the basis of the number of viable bacteria in the untreated control After sufficient time for adsorbtion and inactivation of unadsorbed phage with antiserum, the infected bacteria were plated on appropriate indicator bacteria Adsorbtion was 90 99 per cent in all the experiments

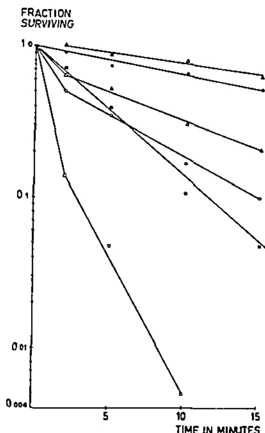


Fig 3

Inactivation of the ability of *E coli B* to support growth of T2 Survival of capacity = closed symbols △ = 740 atm ○ = 1036 atm □ = 1480 atm
Inactivation of bacterial colony formation (= open symbols) is included in the fig

The results for three different pressures are given in Fig 3 The points are averages of three independent experiments The difference in slope between the curves for capacity and survival of the bacteria at a given pressure is not very great The slope is 1 2–1 5 times greater for survival indicating that capacity is slightly more resistant to the treatment than survival There is, however, a striking difference between the curves in that capacity does extrapolate to 1 0 whereas survival does not The rate of adsorbtion to treated bacteria is the same as to untreated

The Effects of Pressure on Vegetative Bacteriophage

In order to further evaluate the effects of pressure on the ability of the bacteria to support growth of bacteriophage, bacteria infected with T2 were exposed to pressure at various times after infection The technique used in these experiments was one described by *Benzer* The bacteria were grown in broth, centrifuged, washed and resuspended in adsorbtion buffer Phage was added at a low moi After proper time for adsorbtion the bacteria were centrifuged and resuspended in buffer

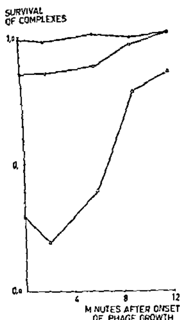


Fig 4

Survival of phage bacterium complexes after treatment with pressure
Time of exposure is two minutes for each point
○ = 740 atm □ = 1036 atm △ = 1480 atm

in order to remove unadsorbed phage The bacteria were then diluted into broth preheated to 37° C circumstances which initiated phage development immediately in most of the infected cells Samples were diluted at intervals into chilled broth which immediately stopped the growth of the phage No increase (or decrease) in titre was observed in the chilled samples if kept 4 hours which was well within the time for one experiment The samples were quickly heated to 37° C and exposed to pressure Immediately after release of the pressure, the samples were again chilled and survival of the infected cells was assayed The assays were performed within 5 10 minutes after release of pressure In samples taken later than 12 13 minutes following dilution into broth phage released during sampling obscured the results The latent period in the controls varied between 19 and 23 minutes and the burst size between 70 and 120 The results are presented in Figs 4 5 6 and 7 Fig 4 shows the effects of 2 minutes exposure to different pressures As no significant difference in sensitivity was found during the first 5 7 minutes, attention was focussed on those times between 7 minutes and the appearance of intracellular mature phage (Figs 5–7) The survival of the bacterium bacteriophage complexes is plotted against that time in the latent period at which the sample was taken The points in the curves are averages of three independent experiments Samples

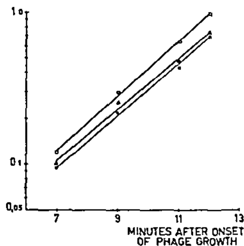


Fig 5

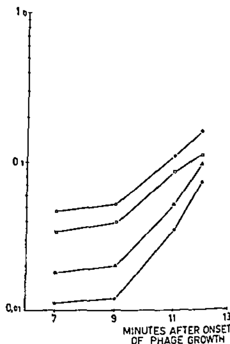


Fig 7

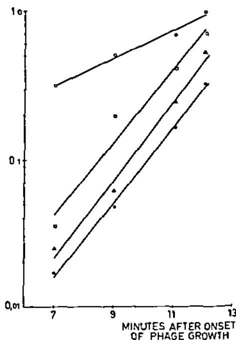


Fig 6

Figs 5–7

Survival of infected complexes after treatment with pressure
Pressure in Fig 5 = 1180 atm Fig 6 = 1200 atm Fig 7 = 1330 atm
Time of exposure is constant for each line
○ = 1 minutes □ = 2 minutes ▲ = 4 minutes ● = 6 minutes

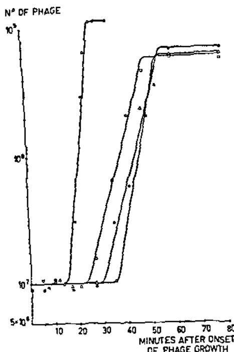


Fig 8

Infected bacteria were treated with 740 atm for 2 minutes various times after the onset of phage synthesis
□ = 0 minutes ● = 7 minutes and Δ = 12 minutes after onset of phage synthesis

were taken at 7, 9, 11 and 12 minutes after dilution into broth Between 10 and 12 minutes intracellular mature phage begins to appear, as judged by release of phage by premature lysis of the cells with chloroform The average number of phage particles found in the bacteria after lysis with chloroform at 11 minutes is 1 2 per cent of the control burst

As can be seen in Figs 5–7, the survival of the complexes increases with time elapsed after the onset of phage growth

The Effect of Pressure on the Burst Curve

Finally, the effect of pressure on the burst curve was studied The technique of infection and sampling was the same as used in the experiments with vegetative phage growth After exposure to pressure for 2 minutes the samples were diluted into 37° C broth and assayed for phage at intervals One such experiment is presented in Fig 8 It can be seen that the burst size is diminished and the latent period prolonged at 2 min exposure to 740 atm This pressure was used because there is very little, if any, loss of infected bacteria

If the latent period is measured from the time the samples are diluted into broth after exposure to pressure, it is found to vary little between

the samples and the control The effect of further increasing the pressure or prolonging the time of exposure simply results in decreasing the burst size until finally there is none or one too small to be measured

DISCUSSION

The inactivation of the bacteria with pressure is exponential but the curves do not extrapolate to 1 0 This effect is probably dependent on the amount of pressure applied, not on the time of exposure Most likely it is an effect of the application (or release) of pressure If the bacterial survival curves are compared with the curves for the capacity of the bacteria to support growth of T2 it is seen that the slope of the curves for survival and capacity are not very different The capacity curves, however, extrapolate to 1 0 After treatment the bacteria probably contain two major fractions One has lost its ability to divide but not to support growth of bacteriophage T2 The other fraction has lost both of these properties Nothing is known of the nature of the damage caused to the bacteria We can, however, assume that the cells having lost their capacity are severely injured, considering the high resistance of the capacity to *e g* ultra-violet irradiation (*Anderson* 1948) It is neither likely that the cells have lysed during the treatment considering the fact that the capacity curves extrapolate to 1 0

The effects of pressure on vegetative phage were undertaken to see whether a situation similar to that for ultra-violet irradiation would be found The resistance of intracellular T-even phage to UV increases during the first 5 10 minutes following infection to a level much too high to be accounted for by the number of phage equivalents contained in the infected cell (*Benzer* 1952, *Luria* & *Latarjet* 1947) After about seven minutes of phage growth there is an increasing resistance to pressure This increase proceeds exponentially, at least between 1180 and 1260 atmospheres and is probably a reflection of the state of maturity of the phage reached within the infected cell at a given time The extrapolation of the curves to 100 per cent survival would then equal the time when all the infected cells contained at least one phage particle in a state resistant to the pressure used If this state was equal to the appearance of mature phage particles, as judged by premature lysis with chloroform, all the curves would extrapolate to roughly the same time Since extracellular T2 is less affected by the pressures used in the experiments (*Rutberg*, unpublished) one is led to assume that intracellular mature phage is more sensitive to pressure than extracellular phage It has actually been proposed by *Kellenberger* that the phage parts seen in *e g* proflavine lysates are degradation products of phage For technical reasons it is not possible to test the sensitivity of "late" complexes in the system used so there always exists the possibility that the curves break very sharply when all the cells contain mature phage

The early experiments of *Foster & Johnson* showed that pressure decreased the burst size of T2 infected cells and also increased the latent period in proportion to the length of exposure We have confirmed their observations on the effects on the burst size Of interest, however, is our finding that the burst size is independent of the time in the latent period the sample is taken and furthermore that the latent period is prolonged in proportion to the time during the latent period at which the cells are exposed to pressure The latent period for the samples exposed to pressure 0 7, and 12 minutes respectively is roughly the same measured from the time of dilution into broth after treatment

Two points seem clear from these experiments One is that the cells when treated during the first 12 minutes of infection have to start making phage anew following pressure treatment Otherwise one would expect *inter alia* a higher burst in the sample taken at twelve minutes as compared to the sample taken at zero minutes The second point is that lysis of the cells is inhibited by the pressure or the cells would be expected to lyse at the same time as the control

The fresh start made by the bacteria either means that all the material necessary for the formation of the phage is destroyed (but not the information for its synthesis) or that some early, critical product is inactivated by the pressure and the synthesis is sequential *e g* the synthesis has to start anew from the point of interruption If the effect were just one of slowing down phage production it is unlikely that the burst size would be about equal for samples taken at those different times

SUMMARY

The effects of high hydrostatic pressure on the viability and the ability to support growth of bacteriophage T2 of *Escherichia coli* has been studied Capacity and reproducibility have been found to be inactivated at a roughly similar rate The sensitivity of intracellular phage is dependent on the state of maturation reached within the infected cell There is some indication that intracellular mature phage is more sensitive to pressure than extracellular phage Finally pressure decreases the burst size and increases the latency period in proportion to the time after infection at which the cells are exposed to pressure

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ON THE EFFECTS OF HIGH HYDROSTATIC PRESSURE ON BACTERIA AND BACTERIOPHAGE

2 *Inactivation of Bacteriophages*

By

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Received 11 x 63

In a previous paper, the effects of high hydrostatic pressure on *Escherichia coli* strain B and its ability to support the growth of bacteriophage T2 were described (*Rutberg* 1964) The present paper is concerned with the inactivation of bacteriophages with high hydrostatic pressure

Basset and co workers have studied the effects of high hydrostatic pressures on a number of biological materials but their data give little indication of the actual type of damage caused by pressure or the kinetics of pressure action *Foster, Johnson & Miller* found that the T phages with the exception of T7, were protected to some extent from thermal inactivation if they were exposed to the elevated temperature at an increased hydrostatic pressure In a study on Tobacco mosaic virus it has been found that TMV protein is denatured at very high pressures (about 7000 atm) but retains its activity in the pressure ranges used in the present study (*Lauffer & Dow* 1942) Recently, marked differences in sensitivity between different strains of influenza virus to hydrostatic pressure has been demonstrated (*Overman & Lewis* 1959) The difference is most marked between influenza A and B

MATERIALS AND METHODS

The bacteriophages used in the present work are T2 T4D T4D r 73 and T5 The phage stocks were all prepared with E coli B as host b [illegible]
stored at 4° C [illegible]
assays were [illegible]
formed as de [illegible]
f pressure [illegible]
treatments v [illegible]

This work has been supported by the U.S Public Health Service Research Grant C-5739 from the National Cancer Institute Public Health Service Bethesda Md

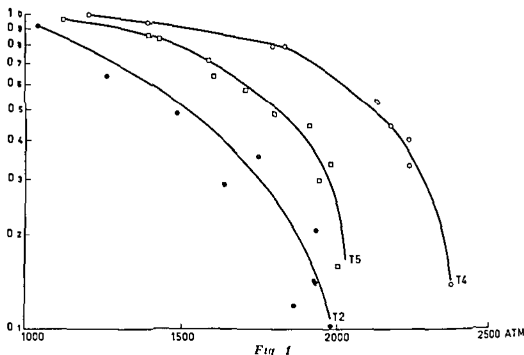


Fig 1

The inactivation of bacteriophages T2 T4, and T5 at different pressures The time of exposure was constant, 5 minutes The experiments were performed at 37° C. Survival was measured on *E coli B*

EXPERIMENTAL

Bacteriophages T2, T4D, and T5 were diluted, from stocks, into broth to give titres ranging from 1 5 × 10⁸ phages/ml and pressure-treated The treatments were carried out with the phage suspended in broth in order to avoid surface denaturation, which might occur in buffer if some air had failed to escape the pressure-tubes (*Adams* 1947–48) The phages were exposed to selected pressures for 5 minutes and surviving phage was assayed immediately after treatment The titre of a pressure-treated preparation has, however, not been found to change after more than one month's storage at 4° C These experiments show that there are marked differences in the pressure sensitivity of the three phages (Fig 1) The relative order of sensitivity to pressure between the three phages is the same as for ultra-violet irradiation (UV) (*Bertani* 1958)

T2 and T4 were chosen for a somewhat more detailed study These phages were exposed to selected pressures for various periods of time and surviving phage measured Logarithmically growing *E coli B* was later infected with the treated preparations at a multiplicity of infection of 2 After adsorbtion, survival of the bacteria was determined by spreading dilutions on plates previously impregnated with anti-phage-serum to avoid reinfection on the plates Assuming the rate of adsorbtion to be the same for treated and control phage, the multiplicity of "killing" phage was calculated The results show that "killing" is in-

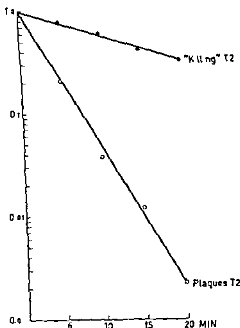


Fig 2

The inactivation of plaque forming ability and "killing" of T2 at 1930 atm Titre killing phage was calculated from the expression fraction surviving bacteria = e x where x = effective multiplicity Adsorbtion was assumed to be equal in the pressure treated samples and in the control

activated at a considerably slower rate than the ability to form a plaque The difference in sensitivity between T2 and T4 is confirmed (Figs 2 and 3)

As the previous experiments had shown that pressure inactivated phage can absorb onto sensitive bacteria it was of interest to see whether the inactivated phage could also inject genetic material In order to test this possibility, 'marker rescue experiments were performed (*Stahl* 1959)

T4D was exposed to pressure and surviving phage titrated The treated phage was allowed to infect *E coli B* at a low multiplicity The bacteria were subsequently superinfected with an rII mutant of T4D (T4D r73) at a multiplicity sufficient to infect all bacteria After inactivation of unadsorbed phage with anti serum, infective centers were scored on *E coli K* lysogenic for phage lambda This host does not permit growth of T4D rII (*Benzer* 1955) Among the infected *E coli B*, only those containing at least one wild type phage in the burst will be scored as infective centers The results indicate that a proportion of the inactivated phages do contribute genetic material in a mixed infection (are "marked rescued) (Table 1)

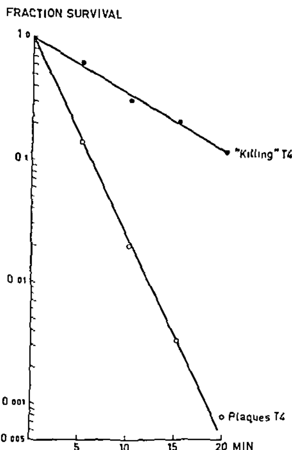


Fig 3

The inactivation of plaque forming ability and 'killing' of T4 at 2220 atm. The experiment was performed the same way as described at Fig 2

TABLE 1

Marker Rescue of Pressure Inactivated T4D

Treatment	Fraction T4D surviving	MOI T4D viable phage	MOI T4D r73 in superinfection	No infectious centers on K(λ) Expected[1]	Found
1 atm	1.0	0.51	5.8	1.6×10^{7}	1.6×10^{7}
5 × 2220 atm	0.41	0.21	5.1	7.6×10^{6}	1.0×10 [illegible]
10 × 2520 atm	0.007	<0.005	6.0	1.6×10^{5}	6.0×10 [illegible]
10 × 2700 atm	0.0005	<0.001	3.7	5.3×10^{4}	3.7×10 [illegible]

T4D was exposed to pressure as indicated and survival measured on *E coli B*. *E coli B* was later infected at an effective moi of 0.5 and subsequently superinfected with T4D r 73 at the moi indicated. Adsorbed phage was measured from input phage and unadsorbed phage, the latter measured by centrifugation of a diluted sample of the infected bacteria. Unadsorbed phage was inactivated by treatment with T4 anti serum for 5 minutes (K = 4.5). Dilutions were plated on K (λ) before lysis of the infected cells and infectious centers scored on this host. Bacterial titre = 4.3×10^{7}. Control T4D r 73 infecting *E coli B* at a moi of 6.3 gave 4.1×10^{4} plaques on K (λ). 1) Expected number of infectious centers was calculated from input number of viable T4D assuming no marker rescue

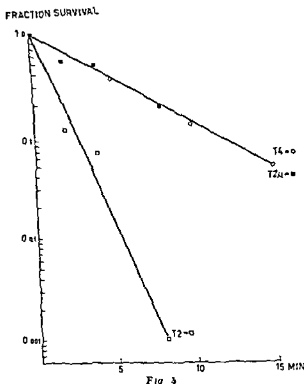


Fig 4
The inactivation of T2 T2u and T4 at 2220 atm

A similar type of experiment has been performed with a host range mutant of T2 Essentially the same type of result was achieved

As the relative order of sensitivity of T2, T4 and T5 is the same as for UV and as marker rescue can be demonstrated with pressure-inactivated phage, the pressure sensitivity of a hybrid of T2 was tested This hybrid was obtained from a cross between T2 and T4, selecting for the UV sensitivity of T4 and some marker of T2 The recombinant has been backcrossed 9 times with T2 always selecting for the UV sensitivity of T4 and some marker T2 (*Streisinger* 1956) This phage, T2 u, was found to have the serological and host range specificity of T2 but the UV sensitivity and the pressure sensitivity of T4 (Fig 4)

DISCUSSION

The pressures required to inactivate free bacteriophage are as expected (*Bassel et al* 1935), considerably higher than those required to inactivate bacteria (*Rutberg* 1964) The sensitivity of the phage to inactivation with pressure is not determined by the morphological characteristics of the phage, as T2 and T4 differ considerably in their relative sensitivities to pressure The primary mode of action of pres

sure is not an inactivation of the ability of the phage to attach to sensitive bacteria, since "killing" is inactivated much slower than plaque forming ability. The fact that a hybrid of T2, having the serological specificity of T2, is less sensitive than wild type T2 also speaks against the possibility that denaturation of phage protein is of importance at the pressures used.

A small fraction of the pressure-inactivated phage can contribute genetic material in a mixed infection. This points to the possibility that *pressure might damage the genetic material of the phage*, as obviously the inactivated phage has to be able to inject in order to contribute a marker in the mixed infection. Whether the main bulk of the inactivated phage does inject or not is not known.

An interesting suggestion emerges form these experiments, namely *that the factors determining the sensitivity of phage to UV and to high hydrostatic pressure coincide*. This suggestion is based on the following findings:

(a) the relative order of sensitivity to pressure among T2, T4, and T5 is the same as for UV, (b) a small fraction of pressure-inactivated phage can contribute genetic material in a mixed infection, (c) a variant of phage T2, having the serological and host range specificity of T2 but the UV sensitivity of T4, has the same sensitivity to pressure as T4.

Experiments performed with uninfected and phage-infected becteria have not lent any support to the idea that pressure should mimic the action of UV (*Rutberg* 1964). Thus the capacity of pressure-treated *E. coli B* to support growth of T2 is roughly equally sensitive to pressure as the colony forming ability. Vegetative T2 does not show the sensitivity pattern to pressure which is typical of UV. The pressure- sensitivity of a UV-resistant strain of *E. coli B* (B/r), is the same as for the ordinary *E. coli* strain B (*Holme* unpubl.).

It has been demonstrated that pressure will induce lysogenic bacteria (*Rutberg* 1964) but the characteristics of this induction seem to be quite different from those of UV.

A likely proposal therefore seems to be that, with respect to extracellular phage, the effects of pressure often coincide with those for UV and the same factors may determine the sensitivity of a phage to the two agents. Bacteria, on the other hand, do not show a similar pattern. This is obviously related to the different pressure levels that have to be used to achieve an effect in the two cases.

This suggestion is in line with the generally accepted idea that there is a profound difference between the mode of action of hydrostatic pressures on biological material below and above 1000-1500 atm (*Johnson et al.* 1954).

SUMMARY

The inactivation of bacteriophages T2 and T4 with hydrostatic pressure has been studied Inactivation of both phages at constant pressure is exponential with time "killing" is inactivated much slower in both phages Marker rescue of pressure inactivated T4 is demonstrated The similarities between the action of ultraviolet light and high hydrostatic pressure on bacteriophage is pointed out

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ON THE EFFECTS OF HIGH HYDROSTATIC PRESSURE ON BACTERIA AND BACTERIOPHAGE

3 *Induction with High Hydrostatic pressure of* Escherichia coli K *Lysogenic for Bacteriophage Lambda*

By

LARS RUTBERG

Received 11 x 63

Bacterophages can be divided into two main classes, temperate and virulent When a virulent bacteriophage infects a sensitive bacterium it reproduces itself in that bacterium A certain period of time after infection, the bacterium lyses and liberates a burst of new phage The temperate phage, however, has two alternatives when infecting a bacterium One is the one obligatory for virulent phage, the other one leads to the condition known as lysogeny When establishing lysogeny the phage does not kill the bacterium but remains in a non-infectious form, the prophage, in close connection with the bacterial chromosome, and behaves in many respects as a bacterial gene The prophage divides in synchrony with the bacterial chromosome, and it confers some new properties on the bacterium The most important of these properties is immunity to superinfection with homologous phage (*Lwoff* 1953, *Bertani* 1958)

In a lysogenic population there is a certain low probability that the balance between the prophage and the bacterium will be upset As a result the prophage will be converted to vegetative phage, reproduce itself and finally the bacterium lyses and liberates a number of mature phages, identical in type with the one which originally infected it This probability is a characteristic of most lysogenic systems (*Six* 1958) The phenomenon itself is known as induction (*Lwoff* 1953, *Jacob* 1954) The frequency of induction can be very much increased by treating the bacteria with a number of agents such as ultra-violet irradiation, nitrogen mustard, peroxide etc All of these agents are potent mutagens and/or carcinogens (*Lwoff* 1953), and possibly act by interfering with the genetic material of the cell Induction can also be achieved by interfering with the nucleotide metabolism of the cell (*Melechen & Skaar* 1960, *Korn & Weissbach* 1962, *Ben-Gurion* 1962) The present commu-

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nication will present evidence that induction of lysogenic bacteria can also be achieved with high hydrostatic pressure, an agent not known to be mutagenic (*Holme* 1963 unpubl, *McElroy & de la Haba* 1949)

MATERIALS AND METHODS

kindly provided by Doctor *G Bertani* In one experiment another strain of E coli K lysogenic for lambda was used This strain was kindly provided by Doctor *E L. Wollman*

The bacteria were grown in 0 8 per cent Difco nutrient broth with 5 grams NaCl added per litre and pH adjusted to 7 4 before autoclaving Phage and bacteria were assayed on plates containing 10 grams Difco tryptone, 10 grams Difco agar and 5 grams NaCl per litre The same medium with 7 grams agar per litre was used as top layer agar

The titrations of phage were done as described by Adams (*Adams* 1959) Viable counts were made by spreading dilutions on the surface of the plates with sterile glass rods All experiments unless stated in the text were performed at 37° C

The bacteria were routinely grown and prepared in the following manner for all experiments concerning induction An overnight culture grown aerobically on a shaker without any additional aeration was diluted 1 200 into fresh broth and put on the shaker until a density of 5×10^7 bacteria/ml was reached The culture was spun down in the cold and resuspended in 1 to ½ the original volume of fresh medium 1 2 ml samples were distributed into small glass vials which were sealed with silicon oil The vials were then put under pressure Controls were treated the same way omitting the pressure The pressure apparatus has been described in an earlier paper (*Rutberg* 1964)

EXPERIMENTAL

K 39, grown and prepared as described, was exposed to pressure Immediately after treatment the bacteria were diluted 1 50 into broth at 37° C and kept at this temperature with aeration At intervals assays were made for viable bacteria and for free phage The latter were determined by plating on K 51 with streptomycin added The results of one such experiment are given in Fig 1 The number of free phage in the pressure treated sample remains essentially constant for the first 60 minutes after dilution into broth There is a very rapid increase in this number during the following hour, at the end of which the number of free phage has risen to a value exceeding the zero time value by 10^4 A plateau is reached and the number of free phage remains roughly constant for the next 60 minutes The number of free phage in the control is considerably less, and the rate of production of phage is roughly equal to the growth rate of the bacteria The pressure treated bacteria do not increase in number during the first hour of incubation after the treatment After this time they start growing at a rate similar to that of the conrol This experiment shows clearly that the bacteria have been induced by the exposure to pressure Extracellular phage lambda is not affected by any of the pressures used

The number of induced bacteria was subsequently determined with the aid of a technique originally developed by Six (*Six* 1959) After

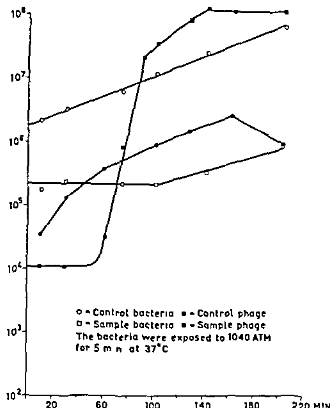


Fig 1

K 39 grown and treated as described in *Materials and Methods* was exposed to 1040 atm for 5 minutes at 37° C. After treatment the bacteria were diluted 1 50 into 37° C broth and aerated At intervals assays were made for the number of viable bacteria and free phage At this pressure there is some killing of K 39 without concomittant production of phage

pressure-treatment, the bacteria were plated with K 51 as indicator bacteria but without any streptomycin added The plates were incubated at 37° C for a certain length of time This time was determined so that all the bacteria that had been induced by the treatment had lysed on the plates The plates were then taken out of the incubator and sprayed with streptomycin After spraying, the plates were incubated one hour at 4° C to let the streptomycin soak through They were then incubated over night at 37° C The number of induced bacteria is thus equal to the difference between the counts on plates with the treated bacteria and the control plates The frequency of induction has been measured with this technique for a number of pressures and a number of times of exposure (Fig 2) The number of bacteria induced at a constant pressure is directly proportional to the time of exposure to pressure As a matter of control, another strain of *E coli K* lysogenic for lambda has been tested It has been found to be equally inducible by pressure

This technique has permitted a demonstration of an inducing effect at 37° C at as low pressure as 290 atm It is possible, however, with a method developed by Marcovich (*Marcovich* 1956), to demonstrate a

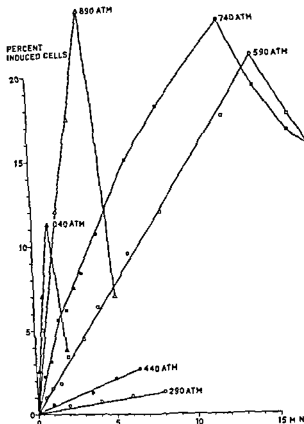


Fig 2

The rate of production of Lambda by K 39 as a *function of exposure time at different* pressures The experimental technique is described in the text

slight inducing effect of a 2 minute exposure at 150 atm No estimate of percentage induction can be made with this technique

The experiments described have all been performed with the bacteria suspended in broth Induction with pressure has also been demonstrated with the bacteria suspended in phosphate buffer during treatment but the effect is repeatedly somewhat less under such conditions Pressure has also been found to induce bacteria starved for 2 hours in a phosphate buffer Bacteria treated in this manner have lost their aptitude for induction with UV (*Lwoff* 1953)

The previous experiments were all performed with pressure-treatment at 37° C The same type of experiment, using the technique of Six has been performed with the pressure treatment at 30° C At this temperature also the number of induced bacteria is directly proportional to time of exposure at constant pressure The rate of induction at both 30° C and 37° C increases exponentially over a pressure range of

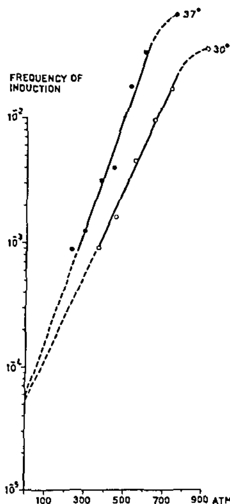


Fig 3

The rate of induction at 30° C and 37° C The experimental technique is described in the text

250 to about 900 atm (Fig 3) The order of magnitude of the volume change of activation can be calculated from these curves It was found to be about 210 cc/mole which indicates that the effect is on some macromolecular component of the bacterium (*Johnson et al* 1954)

It is possible that the capacity of the bacteria to produce bacteriophage is affected by the pressures used (*Rutberg* 1964) Two types of experiments have been performed to measure such an effect This was done bearing in mind particularly that a decreased capacity might be responsible for the declining parts of the induction curves of Fig 2 In one experiment, sensitive bacteria were infected with lambda and submitted to pressure (Fig 4) In the other, lysogenic bacteria were induced with UV and then exposed to pressure (Fig 5) The results of the experiments do not lend much support to the idea that the lowering of capacity is responsible for the declining parts of the induction curves in Fig 2

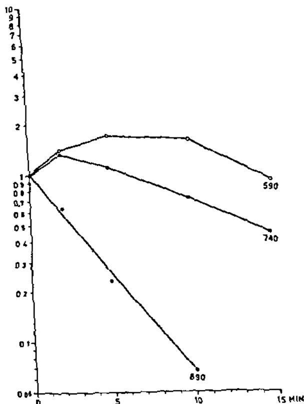


Fig 4

The effect of pressure on the capacity of UV induced bacteria K 39 at a concentra

The number of infectious centers is determined by spraying The pressures are indicated in the figure

DISCUSSION

The experiments presented in this paper have clearly demonstrated that *E coli K*, lysogenic for bacteriophage lambda, is induced by exposure to high hydrostatic pressures The frequency of induction has been found to be directly proportional to the time of exposure at a constant pressure This excludes the possibility that the induction effect is merely due to the application of release of pressure Induction with pressure has hitherto been found to differ from induction with ultra violet light in two respects The dose response is linear for pressure but two hit for UV (*Marcovich* 1956) Bacteria that have lost their aptitude for induction with UV retain their aptitude for induction with

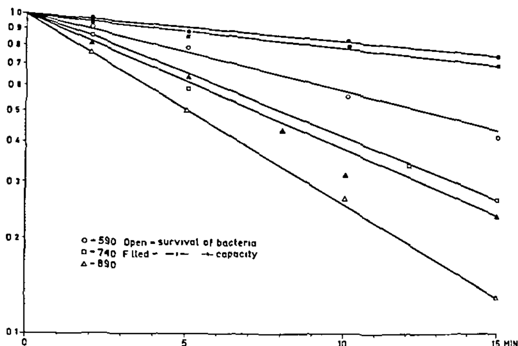


Fig. 5

The effect of pressure on the survival of K 38 and its capacity to support growth of phage lambda. K 38 at a concentration of 3.3×10^8 was infected with lambda at an effective multiplicity of 0.03. Immediately after adsorbtion the bacteria were exposed to pressure as indicated in the figure. After treatment the survival of the bacteria and of the infective centers was determined.

pressure. Although this is not to be taken to mean that the basic mechanism of induction is different in the two systems, it points to the possible use of pressure as a new tool in the study of the induction phenomenon. The possibility that peroxides, which might form during the pressure-treatment, are responsible for the induction, can be excluded, as phage lambda is not inducible with such agents (*Lwoff* 1953).

There are two possibilities which seem to justify a closer investigation of induction of lysogenic bacteria with pressure: (a) the differences between pressure and UV point to the possibility that pressure might provide new information about the practically unknown induction phenomenon, and (b) pressure might provide a tool for induction of previously uninducible phages (*Rutberg & Heden* 1961). Experiments along these lines are now in progress in our laboratory.

SUMMARY

An inducing effect of hydrostatic pressure on *E. coli K* lysogenic for bacteriophage lambda is demonstrated. The rate of induction is dependent on the pressure used. At constant pressure the number of in-

duced bacteria is directly proportional to time of exposure Induction can be achieved both with actively growing and with starving bacteria The possible use of this system is briefly discussed

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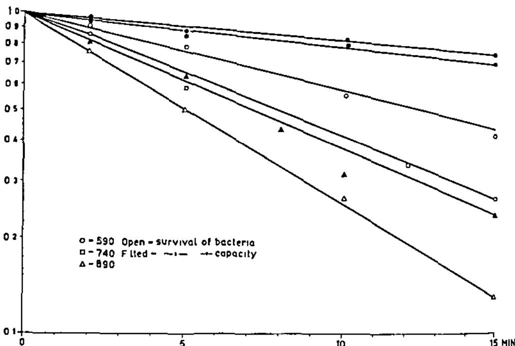


Fig 5

The effect of pressure on the survival of K 38 and its capacity to support growth of phage lambda K 38 at a concentration of 3 3 × 10^8 was infected with lambda at an effective multiplicity of 0 03 Immediately after adsorbtion the bacteria were exposed to pressure as indicated in the figure After treatment the survival of the *bacteria and of the infective centers was determined*

pressure Although this is not to be taken to mean that the basic mechanism of induction is different in the two systems, it points to the possible use of pressure as a new tool in the study of the induction phenomenon The possibility that peroxides, which might form during the pressure-treatment, are responsible for the induction, can be excluded, *as phage lambda is not inducible with such agents* (*Lwoff* 1953)

There are two possibilities which seem to justify a closer investigation of induction of lysogenic bacteria with pressure (a) the differences between pressure and UV point to the possibility that pressure might provide new information about the practically unknown induction phenomenon, and (b) pressure might provide a tool for induction of previously uninducible phages (*Rutberg & Heden* 1961) Experiments along these lines are now in progress in our laboratory

SUMMARY

An inducing effect of hydrostatic pressure on *E coli K* lysogenic for bacteriophage lambda is demonstrated The rate of induction is dependent on the pressure used At constant pressure the number of in-

Fig 1

The effect of four different quaternary ammonium compounds on the potency of poliovirus vaccine (type 1) after periods of storage at +32° C Ordinate potency in ^{10}logs (extinction limit) Abscissa storage time in weeks

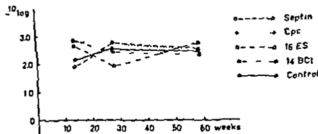


Fig 2

Potence stability at +4° C All other test conditions as in Fig 1

Two series of experiments were made one at normal storage temperature (+4° C), the other at a higher temperature (+32° C) in order to accelerate any inactivating effect on the potency of the vaccine

The various preservatives were tested on a single batch of poliovirus vaccine They were added simultaneously to separate portions of this vaccine, the final concentration of each compound being 1/40 000 Diluent alone was added to the control vaccine

Each mixture was then kept at the respective temperatures and samples were withdrawn for determination of potency on a predetermined time schedule

The determination of potency was done by the "guinea pig method" (*B Melén et al.*, Arch ges Virusforsch VII 4 (1957) and IX 1 (1959) Samples collected at the same time were tested simultaneously

Acta path et microbiol scandinav *61*, 106–108 1964

The State Bacteriological Laboratory Stockholm

THE USE OF QUATERNARY AMMONIUM COMPOUNDS AS PRESERVATIVES IN POLIOVIRUS VACCINE

Preliminary Report

By

L ARRO

Received 21 x 63

The present investigation was made in order to study the influence on the potency of poliovirus vaccine exerted by various quaternary ammonium compounds when used as preservatives (*i e* as antiseptics) in the vaccine

A comparison was made between vaccines to which these compounds had been added, both *inter se* and in relation to a control vaccine without preservative

MATERIALS AND METHODS

Four quaternary ammonium compounds have been tested

1 Benzethonium chloride—10 per cent solution from AB Pharmacia Uppsala

$$\left[CH_3-\underset{CH_3}{\overset{CH_3}{C}}-CH_2-\underset{CH_3}{\overset{CH_3}{C}}-C_6H_4-OCH_2-CH_2-OCH_2-CH_2-\underset{CH_3}{\overset{CH_3}{N}}-CH_2-C_6H_5\right]^+ Cl^-$$

2 Cetylpyridinium chloride—crystalline compound from AB Recip Stockholm

$$\left[C_5H_5N-C_{16}H_{33}\right]^+ Cl$$

3 Quarton 16FS—crystalline compound from Liljeholmens Stearinfabriks AB Stockholm

$$\left[C_{16}H_{33}-\underset{C_6H_5}{\overset{CH_3}{N}}-CH_3\right]^+ CH_3SO_4$$

4 Quarton 14BCl crystalline compound from Liljeholmens Stearinfabriks AB Stockholm

$$\left[C_{14}H_{29}-\underset{CH_2-C_6H_5}{\overset{CH_3}{N}}-CH_3\right]^+ Cl^-$$

Acta path. et microbiol. scand. 61: 109–116, 1964

The University of Bergen School of Medicine, The Gade Institute, Department of Microbiology, and Department of Pathology, Bergen, Norway

INTRADERMAL VERSUS SUBCUTANEOUS IMMUNIZATION WITH TYPHOID PARATYPHOID A AND B VACCINE IN HUMAN ADULTS

1. *Antibody Response*

By

TH. M. VOGELSANG, P. WETTELAND and A. WORMNES

Received 12.x.63

Since anti-typhoid vaccination was introduced at the turn of the century by *Pfeiffer & Kolle* (9) and *Wright & Semple* (12), this form of prophylactic vaccination has been tried in many countries and in some on a large scale. In the two World Wars the troops were vaccinated against typhoid as well as paratyphoid fever. It is a common view that this prophylactic procedure has reduced the incidence of these diseases considerably.

The vaccine has mostly been prepared from smooth, fully virulent strains killed by heating and preserved with phenol, but a great number of different modifications of the original method have been described. In 1934 *Felix & Pitt* (5, 6) demonstrated a new antigen in *Salmonella typhi*, the Vi antigen. They found this and the corresponding antibody to be of importance in immunity, and that heating or treatment with phenol caused considerable damage to this antigen. *Felix* (2) therefore prepared a new type of vaccine, using alcohol not only for the sterilization of the vaccine but also for its preservation. This new type of vaccine was first used in 1941 by the Emergency Public Health Laboratory Service in England for inoculation of civilians. It was adopted by the Royal Air Force in 1943 and by the British Army in 1944 (3).

It has been common practice to prepare a vaccine which in addition to *Salm. typhi* also contains *Salm. paratyphi A* and *B*. This so-called T.A.B. vaccine has usually been injected subcutaneously. However, this mode of administration is often followed by local and constitutional reactions (1). In order to minimize these side-effects *Siler et al.* (10) have for re-vaccination recommended the injection of a single booster dose intradermally. In the present study we have tried this route in primary vaccination and compared the results with those obtained on subcutaneous administration of the vaccine.

Preliminary Results of Potency Determinations

The 32° series was kept under observation for 31 weeks and tested after 4, 8, 12, 17, 22 and 31 weeks (the control vaccine also after 59 weeks) The neutralization test was performed on type 1 in all samples and on type 3 in the 12-, 17-, 22- and 31-week samples

The 4° series has hitherto been under observation for about 18 months, and 14-, 28- and 59-week results are available

Figs 1 and 2 illustrate the results hitherto The potency is plotted as function of time

Comments on Figures

The various samples have been denoted as follows.

Vaccine + benzethonium chloride (Septin)	Septin
Vaccine + cetylpyridinium chloride	Cpc
Vaccine + Quarton 16ES	16ES
Vaccine + Quarton 14BCl	14BCl
Control vaccine (without preservative)	Control

Fig 1 (a–e) shows the reduction of potency at + 32°C The potency falls slowly but distinctly in all samples, including the control (*i e* containing no preservative) It seems to be least affected in the control, however, and most in the Cpc batch, while 16ES, 14BCl and Septin appear to be equivalent (no significant difference)

Fig 1 illustrates only the test with type 1 The determinations on type 3 show the same tendency to reduction of potency and the same relations between the various compounds as in type 1

Fig 2 shows the potency after storage at + 4°C In this series there are at present only three values for each batch Despite their spread, it would seem that no reduction of potency has occurred in any of the samples

SUMMARY

Four quaternary ammonium compounds added to poliovirus vaccine up to a final concentration of 1/40,000 have in long-time tests proved to have a certain inactivating effect on the potency of the vaccine when stored at + 32°C No such effect was observed when the vaccine was kept at the customary temperature of + 4°C

TABLE 7

Typhoid Vi Agglutinins

Titre	Before vaccination		1 month after vaccination		1-1½ years after vaccination	
	s c (14)	i d (169)	s c (14)	i d (140)	s c (12)	i d (116)
1/20			50 0	18 6	25 –	6 0
1/10		0 6	7 1	12 9	8 3	8 6
1/5		0 6	14 3	14 3	16 7	12 1
≂1/5	100 0	98 8	28 6	54 2	50 0	73 3

TABLE 8

Sera from subcutaneously T A B immunized individuals

Titres	Typhoid		Paratyphoid B	
	H aggl (497)	O aggl (497)	H aggl (497)	O aggl (497)
1/1000	2 2	0 2	0 6	0 2
1/500	3 6		1 4	0 2
1/250	6 8	0 4	3 4	0 4
1/100	26 2	3 6	15 9	3 6
1/50	24 6	4 8	19 9	5 6
≂1/25	36 6	91 –	58 8	90

2 Intradermally Immunized Individuals

One month after completed vaccination more than three quarters of the individuals revealed a serum TH titre of 1/100 or higher, and over 50 per cent a TO response of the same level The antibody content against paratyphoid A was less pronounced Although about half the number of individuals revealed an AH titre of 1/100 or more, 88 per cent of the sera were AO negative in 1 50 dilution The BH agglutinin titres had about the same levels as those of the TH while the BO titres were lower than those of the TO About two fifths of the sera showed a BO titre not higher than the 1/25 level

1 1½ years after vaccination the agglutinin titres had decreased considerably although more than one fifth of the sera still reached a TO titre of 1 100 or more A somewhat smaller number of sera revealed BO titres of corresponding levels, while most of the AO titres were less than 1 50

The *Vi agglutinin titres* were as usually observed on the whole considerably lower than those of the H and O One month as well as 1–1½ years after vaccination with phenolized vaccine the antibody response to this antigen was as expected rather slight

TABLE 4

Paratyphoid A O Agglutinins

Titre	Before vaccination		1 month after vaccination		1–1½ years after vaccination	
	s c (14)	i d (169)	s c (14)	i d (140)	s c (12)	i d (116)
1/1000						
1/500						
1/250			14 3	1 4		
1/100			7 1	2 1	8 3	
1/50		0 6	28 6	8 5		5 2
$\leqq$1/25	100 0	99 4	50 0	88 0	91 7	94 8

TABLE 5

Paratyphoid B H Agglutinins

Titre	Before vaccination		1 month after vaccination		1–1½ years after vaccination	
	s c (14)	i d (169)	s c (14)	i d (140)	s c (12)	i d (116)
1/1000		.	7 1	9 3	8 3	0 9
1/500			21 4	18 6		2 7
1/250	7 1	0 6	21 4	34 4	16 7	3 4
1/100		1 2	7 1	22 8		12 8
1/50	14 3	3 6	35 7	12 8	16 7	22 4
$\leqq$1/25	78 6	94 6	7 1	2 1	58 4	57 8

TABLE 6

Paratyphoid B O Agglutinins

Titre	Before vaccination		1 month after vaccination		1–1½ years after vaccination	
	s c (14)	i d (169)	s c (14)	i d (140)	s c (12)	i d (116)
1/1000						
1/500			14 3	6 4		
1/250			50 0	12 1	8 3	3 5
1/100		1 2	28 6	14 3	25 0	13 8
1/50		8 3	7 1	29 3	16 7	19 8
$\leqq$1/25	100 0	90 5		37 9	50 0	62 9

response may in part be due to a slow rate of absorption from the skin, it seems more likely that it may be caused by a local stimulation of antibody production, presumably by the reticulo endothelial cells

One month after vaccination 4/5 of the intradermally injected individuals had a serum AH titre of 1/50 or higher, but the next examination carried out after 1 1½ years showed that the titre had decreased to 1/25 or less in 86 per cent of the vaccinated There was a poor response of AO agglutinins one month as well as 1–1½ years after intradermal immunization The number of corresponding subcutaneous vaccinations in the present work is rather small for definite conclusions The poor antibody response may depend on the *Salm paratyphi A* strain used in the vaccine, or the number of these bacilli may have been too small

As already mentioned, treatment with phenol for killing the strains used in our vaccine is known to cause considerable damage to the Vi antigen However, not far from 50 per cent of the sera showed a weak agglutinin response to this antigen one month after vaccination, and reaction was still demonstrable in 1/4 of the sera 1–1½ years after immunization However, the highest titre obtained was not more than 1 20 The P H L S Report (8) states that although many workers have found the Vi agglutination test satisfactory, others claim that it produces too high a percentage of false positive reactions

There is at present no conclusive evidence as to the degree of protection which T A B vaccination affords, or the best method of preparing the vaccine Besides measuring of the agglutinin response in man different tests have been carried out in laboratory animals, especially in mice and rabbits But as man is the original source of typhoid infection it is difficult to correlate the results with certainty

In 1953 the Yugoslav Typhoid Comission (14) organized the first strictly controlled field trial of two types of antityphoid vaccine—alcoholized and phenolized—in an attempt to determine the relat [illegible]

[illegible] e degree of protection would be obtained with other batches of either vaccine Laboratory work during the field trial showed that the existing laboratory tests could not at present be correlated with the protection afforded to man

H O and Vi agglutination tests were performed The results showed a clear distinction in the serological responses to the vaccines, as the alcoholized vaccine promoted a significantly higher titre of Vi antibodies while the H and O titres were found to be somewhat higher when phenolized vaccine had been used

Without discussing whether immunity depends on the Vi, O or H antigens or some other substances Ikic (7) has analysed the dose—response regression lines of alcoholized and phenolized vaccines, ex

3 *Subcutaneously Immunized Individuals*

None of the agglutinin titres obtained after this route of T A B vaccination revealed conclusive differences when compared with those following intradermal injections of the vaccine On an average slightly higher AO, BO and Vi titres were found in the subcutaneously immunized group one month after vaccination, but following 1–1½ years the agglutinin levels were about the same in both groups

In a previous communication (12), 497 sera had been examined from individuals immunized subcutaneously with T A B vaccine at least 6 months earlier (Table 8) If we regard a dilution of 1/50 as the lower borderline for definite agglutinin response, nine-tenths of the sera gave no demonstrable TO reaction In the remaining sera the TO agglutination was very weak, only two showing a titre of 1/250 and one 1/1000

On the other hand, only about one-third of the sera did not reveal demonstrable TH response Most sera showed weak TH titres, while about 1/8 had an agglutinin titre of 1/250 or more About the same number of sera, which did not show any definite TO response, also lacked demonstrable BO reaction Among the other sera four revealed a BO titre of 1/250 or more, while most of those remaining showed a weak BO response More than half of the sera had a BH titre not exceeding 1/25, while about 5 per cent showed a BH titre of 1/250 or more

DISCUSSION

By comparing the antibody response occurring after intradermal and subcutaneous administration of T A B vaccine in human adults, it seems apparent that the response after intradermal injection is generally slightly less pronounced but as persistent as that after subcutaneous administration One month after intradermal vaccination a good response was observed regarding both TH and BH agglutinins, and about 3/4 of the sera had a TO titre of 1/50 or higher, and about 2/3 a corresponding BO level One to 1½ years after vaccination, the TH and BH titres had decreased to less than 1/50 in 73 and 58 per cent, respectively

Most of the sera whose titres are recorded in Table 8 were examined ½–1 year after subcutaneous vaccination The TH titres of these subcutaneously vaccinated individuals were higher than those of the present intradermally vaccinated On the other hand, 9/10 of the subcutaneously injected individuals revealed TO and BO titres of less than 1/50, while more than 1/3 of the intradermally vaccinated had higher serum titres of these agglutinins

Tuft et al (11) suggest that the good response occurring uniformly after intradermal injection may be evidence of a possible active participation of the skin in the production of antibodies, although obviously this function is not limited to the skin Even though the antibody

response may in part be due to a slow rate of absorption from the skin, it seems more likely that it may be caused by a local stimulation of antibody production, presumably by the reticulo endothelial cells

One month after vaccination 4/5 of the intradermally injected individuals had a serum AH titre of 1/50 or higher, but the next examination carried out after 1–1½ years showed that the titre had decreased to 1/25 or less in 86 per cent of the vaccinated There was a poor response of AO agglutinins one month as well as 1–1½ years after intradermal immunization The number of corresponding subcutaneous vaccinations in the present work is rather small for definite conclusions The poor antibody response may depend on the *Salm paratyphi A* strain used in the vaccine, or the number of these bacilli may have been too small

As already mentioned, treatment with phenol for killing the strains used in our vaccine is known to cause considerable damage to the Vi antigen However, not far from 50 per cent of the sera showed a weak agglutinin response to this antigen one month after vaccination, and reaction was still demonstrable in 1/4 of the sera 1–1½ years after immunization However, the highest titre obtained was not more than 1 20 The P H L S Report (8) states that although many workers have found the Vi agglutination test satisfactory, others claim that it produces too high a percentage of false positive reactions

There is at present no conclusive evidence as to the degree of protection which T A B vaccination affords, or the best method of preparing the vaccine Besides measuring of the agglutinin response in man, different tests have been carried out in laboratory animals, especially in mice and rabbits But as man is the original source of typhoid infection, it is difficult to correlate the results with certainty

In 1953 the Yugoslav Typhoid Comission (14) organized the first strictly controlled field trial of two types of antityphoid vaccine—alcoholized and phenolized—in an attempt to determine the relative and absolute effectiveness of each The phenolized vaccine proved the more effective of the two giving protection to about 70 per cent of the vaccinated but the Commission pointed out that the results do not necessarily mean that the same degree of protection would be obtained with other batches of either vaccine Laboratory work during the field trial showed that the existing laboratory tests could not at present be correlated with the protection afforded to man

H, O and *Vi agglutination* tests were performed The results showed a clear distinction in the serological responses to the vaccines, as the alcoholized vaccine promoted a significantly higher titre of Vi antibodies while the H and O titres were found to be somewhat higher when phenolized vaccine had been used

Without discussing whether immunity depends on the Vi, O or H antigens or some other substances, Ikić (7) has analysed the dose—response regression lines of alcoholized and phenolized vaccines, ex-

pecting to provide some information on whether these vaccines contained only one active substance on which the establishment of immunity depended Based on all testings of alcoholized and phenolized vaccines used in the Yugoslav field trial, he found it evident that these two series contained the same active substance The difference in protection provided by these two typhoid vaccine batches he stated to depend only on varying concentrations of one and the same active substance

SUMMARY AND CONCLUSIONS

Intradermal injections of 0 10, 0 15 and 0 20 ml of a phenolized T A B vaccine given to human adults at intervals of 5–7 days were followed by very slight local and constitutional reactions

The antibody response after intradermal vaccination has, on the whole, been slightly less pronounced but as persistent as that after subcutaneous administration of the same vaccine in slightly higher doses One to 1½ years after intradermal vaccination the O agglutinin contents of the sera were higher than those following subcutaneous injections of the vaccine

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INTRADERMAL VERSUS SUBCUTANEOUS IMMUNIZATION WITH TYPHOID PARATYPHOID A AND B VACCINE IN HUMAN ADULTS

2 Serum Protein Investigations

By

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Received 12 x 63

It is well known that infections are generally associated with an increase in serum γ globulin and that this increase is due to formation of specific as well as non specific antibodies It is uncertain if a constant relation exists between these two components (9) *Gourvitch* (6) has studied by means of isotopes (^{14}C labelled glycine), how the ratio of specific to non specific serum γ globulins changes in immunization He observed that immunization of rabbits with horse serum albumin altered the specific as well as the non specific γ globulin content that these changes were largely independent of each other, and that even during the period of maximum specific γ globulin formation there was no inhibition of non specific γ globulin production He concluded that the formation of specific γ globulins was not linked to the transformation of non specific γ globulins to specific antibodies, nor to a change in the synthesis of non specific γ globulins

Moreover, the quantity of γ globulin is frequently altered under a variety of pathological conditions and it is evident that the total quantity of γ globulin cannot be used as the sole criterion of immunity (13) However besides causing variations in γ globulin typhoid and paratyphoid fever have also been known to cause alterations in the serum total protein albumin and in the α and β globulin fractions (2 3 9 10)

However less mention is found of serum protein alterations connected with typhoid paratyphoid immunization *Laurell* (11) fractionated sera by paper electrophoresis and found that the agglutinins against S typhi H antigens were associated with the γ globulins while the agglutinins against S typhi O antigens migrated faster and were found in the fractions between γ and β globulins (β globulins)

In our preceding communication (16) the results of the agglutinin titrations after typhoid paratyphoid A and B (T A B) vaccination by

the subcutaneous and intradermal route in human adults were given The present paper deals with the simultaneous alterations in the serum proteins in the same subjects

MATERIALS AND METHODS

Fourteen healthy young female adults (trainee nurses) were vaccinated subcutaneously and 155 intradermally with T A B vaccine The preparation of the vaccine as well as the doses number of injections and their intervals were given in the preceding communication (16) Blood was withdrawn immediately before immunization and one month after the third injection Serum was collected centrifuged and after samples had been removed for the agglutinin reactions stored at —20° C until immediately before the determination of total protein and fractionation by paper electrophoresis

One month after the third injection of vaccine serum samples suitable for these analyses were obtained from 104 of the intradermally and all the 14 subcutaneously injected individuals The serum total protein was determined colorimetrically by the *Biuret* copper sulphate method using an Evelyn photoelectric colorimeter with a filter of 560 μ Double analyses were always performed and the mean value calculated as grams per cent of serum

The electrophoretic separation of the serum proteins was carried out according to slight modifications of the well known method of *Grassmann et al* (8) using sodium veronal buffer with pH = 8 6 and a constant current of 1 mA per paper strip for 16 hours 0 010 ml serum was applied to each paper which after separation of the proteins was dried for 10 minutes at 100° C and dyed with Amidoschwartz 10 B The colour not fixed to the proteins was removed with glacial acetic acid in metanol and the process repeated five times until the decolourizing fluid was practically clear

After drying and immersion of the paper strips in α bromnaphtalin/paraffin transparence oil scanning was performed on weight analytic paper with a Zeiss photoelectric extensiometer (Extinktionschreiber) and the baseline drawn as advocated by *Sommerfelt* (15) Perpendiculars were dropped to the baseline from the lowest points between the peaks (12) The areas representing the various fractions were meticulously cut out with a pair of scissors and weighed on a semi automatic Mettler weight with an accuracy of 0 1 mg

The relative amount of each protein fraction was calculated as a percentage of their total weight From these percentages and the serum total protein content the absolute amount of each fraction was determined as grams per cent of serum The differences in total protein albumin globulin (A/G) ratio and the protein fractions before the first injection and one month after the third were calculated for each individual

A statistical analysis has been carried out using Student s t test applied to the method of paired samles (4)

$$t = \frac{\bar{d}}{e_{\bar{d}}} = \frac{\bar{d}\sqrt{n}}{s_d}$$

where $\bar{d}$ = mean difference before ($\bar{b}$) and after (a) vaccination ($\bar{d} = a - \bar{b}$)
$e_{\bar{d}}$ = standard error of $\bar{d}$
n = number of paired observations and
s_d = standard deviation of the individual differences (d)

$$= \pm \sqrt{\frac{\Sigma(d)^2 - \frac{1}{n}(\Sigma d)^2}{n-1}}$$

The probability (P) of statistically significant differences was determined from a table of t values with n' = n 1 degrees of freedom (d f)

Fractions revealing significant differences in absolute and/or relative values were checked 1 1½ years after vaccination when serum samples suitable for analysis

were obtained from 96 intradermally and 10 subcutaneously injected individuals The results were compared statistically with the respective values before immunization all methods being identical to the above mentioned procedures

RESULTS

The average values before and after vaccination, their differences and the probability of significance of these are given in the Tables 1–8

Total Serum Protein

As will be seen from Table 1, an average increase significant at the 1–2 per cent level had occurred in the subcutaneously immunized individuals in the course of the first month after the third injection of T A B vaccine One to 1½ years later a positive but lower difference was also encountered, its probability of significance was only 2–5 per cent (Table 8) In the intradermally injected group however, the differences before and one month after vaccination were considerably lower, and not significant statistically

Albumin Globulin Ratio

Table 1 also shows that no significant alterations were observed with regard to the A G ratio one month after completed immunization, irrespective of method

Albumin Fraction

A slight decrease in the average relative value was found in the subcutaneously injected individuals, and but slight increases in the other figures (Table 2) However, significant differences in relative or absolute values were not observed in any group one month after vaccination

α_1 Globulin Fraction

On an average a relative decrease significant at the 1–2 per cent level was noted both after one month and after 1–1½ years for individuals immunized by the subcutaneous route (Tables 3 and 7)

α_2 Globulin Fraction

After one month a significant increase had taken place with regard to the grams per cent value in the subcutaneously injected individuals only (Table 4) After 1–1½ years, however, the slight positive difference proved statistically insignificant (Table 8) No significant alterations were observed in the other series during the first month, either in the absolute or the relative values

the subcutaneous and intradermal route in human adults were given. The present paper deals with the simultaneous alterations in the serum proteins in the same subjects.

MATERIALS AND METHODS

Fourteen healthy young female adults (trainee nurses) were vaccinated subcutaneously and 155 intradermally with T.A.B. vaccine. The preparation of the vaccine as well as the doses, number of injections and their intervals were given in the preceding communication (16). Blood was withdrawn immediately before immunization and one month after the third injection. Serum was collected, centrifuged and, after samples had been removed for the agglutinin reactions, stored at $-20°$ C until immediately before the determination of total protein and fractionation by paper electrophoresis.

One month after the third injection of vaccine, serum samples suitable for these analyses were obtained from 104 of the intradermally and all the 14 subcutaneously injected individuals. The serum total protein was determined colorimetrically by the *Biuret* copper sulphate method, using an Evelyn photoelectric colorimeter with a filter of 560 μ. Double analyses were always performed and the mean value calculated as grams per cent of serum.

The electrophoretic separation of the serum proteins was carried out according to slight modifications of the well known method of *Grassmann et al.* (8) using sodium veronal buffer with pH = 8.6 and a constant current of 1 mA per paper strip for 16 hours. 0.010 ml serum was applied to each paper, which, after separation of the proteins, was dried for 10 minutes at 100° C and dyed with Amidoschwartz 10 B. The colour not fixed to the proteins was removed with glacial acetic acid in metanol, and the process repeated five times until the decolourizing fluid was practically clear.

After drying and immersion of the paper strips in α-bromnaphtalin/paraffin transparence oil, scanning was performed on weight-analytic paper with a Zeiss photoelectric extensiometer ("Extinktionschreiber") and the baseline drawn as advocated by *Sommerfelt* (15). Perpendiculars were dropped to the baseline from the lowest points between the peaks (12). The areas representing the various fractions were meticulously cut out with a pair of scissors and weighed on a semi-automatic Mettler weight with an accuracy of 0.1 mg.

The relative amount of each protein fraction was calculated as a percentage of their total weight. From these percentages and the serum total protein content the absolute amount of each fraction was determined as grams per cent of serum. The differences in total protein, albumin-globulin (A/G) ratio and the protein fractions before the first injection and one month after the third were calculated for each individual.

A statistical analysis has been carried out, using Student's t-test applied to the method of paired samles (4).

$$t = \frac{\bar{d}}{e_{\bar{d}}} = \frac{\bar{d}\sqrt{n}}{s_d}$$

where $\bar{d}$ = mean difference before ($\bar{b}$) and after (a) vaccination ($\bar{d} = \bar{a} - \bar{b}$)
$e_{\bar{d}}$ = standard error of $\bar{d}$
n = number of paired observations and
s_d = standard deviation of the individual differences (d)

$$= \pm \sqrt{\frac{\Sigma(d^2) - \frac{1}{n}(\Sigma d)^2}{n-1}}$$

The probability (P) of statistically significant differences was determined from a table of t-values with $n' = n - 1$ degrees of freedom (d.f.).

Fractions revealing significant differences in absolute and/or relative values were checked 1–1½ years after vaccination, when serum samples suitable for analysis

band and specific albumin staining may compensate in the range of the method Analysis of normal values in comparable studies has led to a provisional use of an A/G ratio of 1 0 as a threshold for normality (1), and in the present investigation the average normal value was close to 1 2

Intradermal T A B injections caused a slight increase, and immunization by the subcutaneous route a comparable slight decrease in the A G ratio None were significant, however, and with regard to the latter it may therefore be concluded that the increases in α- and γ globulins observed in this series were almost compensated by the simultaneous increase in albumin

Albumin fraction A slight but only relative decrease was found in the subcutaneously injected individuals only, while the other albumin values were slightly increased No differences were significantly large enough to allow definite conclusions

In severe cases of typhoid fever, however, *Benhamou* (3) found a marked drop in albumin *Antweiler* (2) also stressed that the albumin fraction was considerably lowered during the period of continuous typhoid and paratyphoid fever *Herten* (9) observed an initial decrease in serum albumin, and minimal albumin values have been noted as the temperature falls (10), subsequently followed by normalization

α globulins The present study revealed no significant alterations in these fractions when the T A B vaccine had been given intradermally In subcutaneous vaccination a relative decrease in α_1 globulin occurred, while an absolute increase in the α_2 fraction was noted The decrease in α_1 globulin is difficult to explain, while the increase in α globulin parallels the usual findings in typhoid fever *Antweiler* (2) stated that not only the γ globulin but also both α globulin fractions were increased during the period of continuous typhoid and paratyphoid fever

In children suffering from these diseases, *Herten* (9) observed an initial increase in α globulins on micro-electrophoresis (Antweiler's method), followed in the second week by an increase in γ globulin In two cases of severe typhoid fever, electrophoresis, by Tiselius-Longworth s method, revealed a general increase in all globulin fractions and in the fibrinogen (3)

β and γ globulins The alterations within these immunologically closely connected fractions will also be dealt with simultaneously

Ninety-eight *per cent of the immuno globulins* are stated to be found in the γ globulin fraction (5), but some human antibodies, like the typhoid O agglutinins, may be found in the β fraction (9, 11) Though *Antweiler* (2) reported the latter to lack a characteristic response in typhoid disease, an initial but relatively short increase has been noted by *Herten* (9) in 17-20 per cent of children with moderate or severe symptoms Normalization of the β fraction was usually found as the γ globulin started to increase

In the present intradermally immunized individuals a relative, sig

β Globulin Fraction

One month after completed vaccination the intradermally injected group revealed a slight decrease in the relative value only, and with a low probability of significance (Table 5) One to 1½ years after immunization, however, decreases significant at the 0 001 level were found for the average relative as well as the absolute value within the same group (Tables 7 and 8)

γ Globulin Fraction

The only significant differences were found one month after subcutaneous vaccination, when both the average percentage and the grams per cent value of γ globulin had increased in these individuals (Table 6) This fraction was the only one that simultaneously showed significant relative and absolute differences one month after completed immunization with T A B vaccine One to 1½ years later, however, only slighter and insignificant increases were encountered (Tables 7 and 8)

DISCUSSION

Serum Protein Alterations one Month after Completed Vaccination

One month after the last of three weekly performed, subcutaneous injections of T A B vaccine a statistically significant increase was found in serum total protein, α_2 and γ globulin, the latter also revealed an increased relative value A significant but only relative decrease was observed in the α_1 globulin fraction Corresponding, intradermally performed vaccination caused a statistically significant alteration in the β globulin fraction only, and its relative amount was found to be decreased after one month

An over-all slighter response was to be expected in the latter series, as the doses of vaccine were smaller (16) and the clinical reactions generally less pronounced in these individuals

Total serum protein A decrease in serum total protein is generally reported to be associated with typhoid and paratyphoid fever (2, 3, 9, 10), followed by a slow normalization after the third week (9) In the present investigation, subcutaneously performed T A B vaccination caused a significant increase in serum total protein It is to be expected, however, that the total protein response to vaccination in healthy young adults should be different from that in actual, severe disease

Albumin-globulin ratio A relative rise in the globulin concentration may result from reticulo endothelial stimulation, manifesting itself by a decrease in the A G ratio of serum Human ratios determined electrophoretically at pH = 8 6 usually approximate two thirds of the ratios found by salt fractionation methods *Abdel Wahab et al* (1) found that while failure to follow Beer's law leads to underestimation of the electrophoretically determined A/G ratio in most cases, trailing of the albumin

Relation between Serum Protein Alterations and Agglutinin Content against Typhoid Paratyphoid Antigens

In typhoid and paratyphoid fever, a parallel has not been reported between the variations in serum γ globulin and the agglutinin content against the specific antigens (2, 9 10)

If a connection exists between the O antibodies and the β globulin increase reported in typhoid paratyphoid disease (9) it has been concluded that the O agglutinins must increase earlier than the H anti bodies and show an earlier decrease *Herten* (9) stated that this se quence of events takes place in immunization of human beings with T A B vaccine However, this vaccine may have different properties with regard to the H and O agglutinin formation, partly depending on the production method of the vaccine (16)

The present significant increase in the electrophoretically defined γ globulins one month after subcutaneous vaccination may possibly be caused by the use of formalin killed T A B vaccine (16), which pro duced a marked increase in the H agglutinins connected with the "intra gamma electrophoretic area The increase in the O agglutinin response was consistently lower (16), and may account for the lack in demonstrable production of the partly ' intra beta ' located γ_1A glo bulin

SUMMARY

A statistically significant increase was found in serum total protein α and γ globulin one month after the last of three weekly performed, subcutaneous injections of typhoid paratyphoid A and B (T A B) vac cine in healthy human adults The γ globulin fraction was the only one that simultaneously showed a relative as well as an absolute increase, while a significant but only relative decrease was observed in α_1 globu lin It seems possible that the increase in the electrophoretically defined γ globulin may have been caused by the use of formalin killed T A B vaccine in the present case, this vaccine being known to produce a marked increase in the H agglutinins which are found within the elec trophoretic γ area of serum

Corresponding intradermally performed vaccination caused a sta tistically significant alteration in the β globulin fraction only, the rela tive amount of which was found to be decreased after one month It is pointed out that a lower over all response was to be expected in this series as the doses of vaccine were smaller and the clinical reactions generally less pronounced in these individuals

The initial increase in α and γ globulins observed in subcutaneous T A B vaccination did not persist one to 1½ years after immunization By this time the H agglutinin levels had also decreased considerably Conclusions regarding the persistence of the alterations in the other protein fractions could not be drawn

nificant decrease was observed However, this observation may not necessarily be inconsistent with an initial increase in β globulins An increase may have taken place, but, before eventual final normalization, have been followed by a postprimary decrease at the time of the analysis, which was carried out as late as one month after the completed immunization

However, the above-mentioned variations within the electrophoretically defined β globulin area may have been caused by alterations within the γ immuno globulins of serum Recent investigations (14) indicate that in the adult, exclusive of the components of the complement and properdin systems, three major families of circulating immuno globulins are found, which have been designated γ_2, γ_1A and γ_1M The two latter, which are identical with the β_- globulins (β_2A and β_2M) of *Grabar & Williams* (7), migrate faster than the former Typhoid O agglutinins and paratyphoid B antibodies have been identified as belonging to the γ_1A group (14), which is partly found within the electrophoretically defined β area of serum

The typhoid H antibodies do not show the "extra gamma" electrophoretic spread as the O agglutinins, but have the same electric mobility as the electrophoretically defined γ globulins This, from an immunological view-point, dominating fraction was the only one that showed a relative as well as an absolute increase following T A B vaccination, but in the subcutaneously injected group only

Serum Protein Alterations 1–1½ Years after Vaccination

These results are difficult to evaluate, since "silent" infections and minor infectious diseases may have produced "non specific" alterations in the serum protein fractions during this time However, values that did not differ significantly from those before immunization were found regarding the absolute value for α_2 and the absolute as well as the relative figure for γ globulin It may therefore be concluded that in subcutaneously performed T A B immunization the initial increase in these fractions does not persist after one year

By paper electrophoresis (Cremer Tiselius' method), *Huber* (10) observed normalization of the electrophoretically increased γ globulin values after the fall of the temperature in six cases of typhoid fever, irrespective of chloromycetin treatment The late decrease in the presumably antibody containing serum γ globulin was explained by partial fixation of this fraction *in the tissues* ("sessile" antibodies), and the present findings after T A B immunization by the subcutaneous route are not inconsistent with this theory

The persisting alterations in serum total protein, α_1 and β globulin, however, *do not permit* any conclusions with regard to the duration of the alterations observed one month after T A B immunization in human adults

TABLE 4

α₂ globulin Fraction

Amount	Mode of inject	b	a	d	S_d	t_d	P (d=0)
% TP	S c	8 776	9 023	+ 0 247	0 712	1 298	>0 20
	I d	9 113	8 893	— 0 220	1 316	1 706	>0 05
Cm %	S c	0 653	0 696	+ 0 043	0 058	2 786	0 02>P>0 01
	I d	0 718	0 702	— 0 016	0 116	1 432	>0 10

TABLE 5

β globulin Fraction

Amount	Mode of inject	b	a	d	S_d	t_d	P (d=0)
% TP	S c	12 439	12 338	— 0 101	1 148	0.331	>0 70
	I d	12 497	12 217	— 0 280	1 266	2 261	0 05>P>0 02
Cm %	S c	0 927	0 949	+ 0 022	0 090	0 917	>0 30
	I d	0 985	0 966	— 0 019	0 129	1 515	>0 10

TABLE 6

γ globulin Fraction

Amount	Mode of inject	b	a	d	S_d	t_d	P (d=0)
% TP	S c	20 034	20 750	+ 0 716	1 008	2 657	0 02>P>0 01
	I d	20 030	20 089	+ 0 059	1 848	0.324	>0 70
Cm %	S c	1 491	1 603	+ 0 112	0 127	3 311	<0 001
	I d	1 581	1 593	+ 0 012	0 195	0 653	>0 50

Tables 7–8 One to 1½ years after T A B vaccination

10 paired observations in the subcutaneously and 96 in the intradermally immunized series

TABLE 7

Values as per Cent of Total Protein ("Relative" Amounts)

Fraction	Mode of inject	b	a	d	S_d	t_d	P (d=0)
α₁ glob	S c	4 326	3 861	— 0 665	0 715	2 943	0 02>P>0 01
α₂ glob	S c	8 936	9 289	+ 0 352	1 155	0 964	>0 30
γ glob	S c	19 965	20 494	+ 0 579	1 941	0 444	>0 60
β glob	I d	12 590	11 624	— 0 956	1 488	6 297	<0 001

LEGENDS FOR THE TABLES

Analysis of the differences in mean values of serum protein fractions before and after subcutaneous and intradermal immunization with T A B vaccine in human adults

Abbreviations

s c	= subcutaneous vaccination
i d	= intradermal vaccination
T P	= total protein (serum)
% T P	= per cent of T P
Gm %	= grams per cent (of serum)
A/G	= albumin/globulin ratio (electrophoretically determined)
b	= mean value before vaccination
a	= mean value after vaccination
$\bar{d}$	= mean difference (a b)
S_d	= standard deviation of the individual differences (d)
t	= *Student's* t value (number of degrees of freedom given by subtraction of 1 from the numbers given in the legends for the Tables 1 6 and 7 8)
P	= probability of significance of mean difference ($\bar{d}$)

Tables 1–6 One month after the last injection of T A B vaccine

14 paired observations in the subcutaneously and 104 in the intradermally immunized series

TABLE 1
Serum Total Protein and Albumin/Globulin Ratio

T P A G	Mode of inject	b	a	$\bar{d}$	S_d	$t_{\bar{d}}$	P ($\bar{d}$=0)
T P	S c	7 442	7 712	+ 0 270	0 342	2 951	0 02>P>0 01
(Gm %)	I d	7 870	7 908	+ 0 038	0 555	0 702	>0 40
A/G	S c	1 186	1 165	— 0 021	0 112	0 719	>0 40
	I d	1 197	1 219	+ 0 022	0 126	1 793	>0 05

TABLE 2
Albumin Fraction

Amount	Mode of inject	b	a	$\bar{d}$	S_d	$t_{\bar{d}}$	P ($\bar{d}$=0)
% T P	S c	54 176	53 744	— 0 432	2 255	0 717	>0 40
	I d	54 300	54 791	+ 0 491	2 564	1 954	>0 05
Gm %	S c	4 030	4 146	+ 0 116	0 252	1 732	>0 10
	I d	4 269	4 329	+ 0 060	0 335	1 827	>0 05

TABLE 3
α_1 globulin Fraction

Amount	Mode of inject	b	a	$\bar{d}$	S_d	$t_{\bar{d}}$	P ($\bar{d}$=0)
% T P	S c	4 574	4 145	— 0 429	0 600	2 676	0 02>P>0 01
	I d	4 030	4 010	— 0 020	0 833	0 250	>0 80
Gm %	S c	0 340	0 321	— 0 019	0 037	1 934	>0 05
	I d	0 316	0 317	+ 0 001	0 067	0 197	>0 80

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SEPARATION OF RHEUMATOID FACTORS AND ANTISTREPTOLYSINS BY GEL FILTRATION AND PREPARATIVE ELECTROPHORESIS

By

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Received 28 xi 63

Antibodies of 7S γ (γ_{ss}) and γ_{1M} (β_{2M} 19 S γ) globulin type have been demonstrated by several investigators (6, 18 25, 39, 40) γ_{1A} (β_{2A}) globulins have also been claimed to carry antibody activity (6, 8 12, 27 37, 39 40) Antibodies with sedimentation coefficients between 7 and 19 Svedberg units have been found and these are immunologically related to either 7S γ or γ_{1A} globulins (27, 37, 40)

The separation of antibodies has been performed by various physicochemical methods *e g* precipitation techniques, electrophoresis, ion exchange chromatography and ultracentrifugation (6 12 18 25, 27 37, 39 40) The dissociation of specific antigen antibody complexes has also been used for purification purposes (18, 40)

It has previously been established that rheumatoid factor (RF) is a γ_{1M} globulin with a sedimentation coefficient of 19S and that it can bind 7S γ globulins forming complex of about 22S (26) It has recently been claimed that RF of lower molecular size also exists (3 26)

Antistreptolysin (AST) has been associated with γ globulins and unspecific AST activity has been ascribed to lipoprotein fractions in human serum (1 11 15 31)

Filtration on gels (34) such as the cross linked dextran gel Sephadex G 200 which separate large molecules has provided means for analytical and preparative separation of antibodies of 7S γ and γ_{1M} types

The γ_{1M} antibodies are eluted together with the macroglobulins in the void volume and 7S γ globulins are found in the second main protein peak (9, 19 23) Antibodies of intermediate type as judged by its elution from Sephadex G 200 between the 7S and 19S types has also been demonstrated (8 19 23) A high degree of purification of γ_{1M} globulins and 7S γ globulins was obtained by gel filtration followed by preparative electrophoresis (10 19)

This investigation was supported by grants from the Medical Faculty University of Uppsala and the Society for Medical Research Stockholm Sweden

TABLE 8

Values as Grams per Cent of Serum (Absolute Amounts)

Fraction	Mode of inject	b	a	d	S_d	t_d	P (d=0)
T P	S c	7.516	7.799	+0.283	0.365	2.453	0.05>P>0.02
α_1 glob	S c	0.340	0.301	−0.039	0.057	2.162	>0.05
α_2 glob	S c	0.672	0.724	+0.052	0.103	1.601	>0.10
γ glob	S c	1.499	1.601	+0.102	0.196	1.636	>0.10
β glob	I d	0.992	0.909	−0.083	0.139	5.893	<0.001

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antigen In other experiments six cylindrical wells (diameter 4 mm) were arranged around a central hole at 6 mm distance

These techniques were used for qualitative as well as semiquantitative analyses of antigens Each or every second fraction of the eluate was tested before or after concentration Immunoelectrophoresis and diffusion in gel experiments were recorded at 6-12 hour intervals for 48-72 hours The slides were then washed in saline and distilled water dried and stained by amido black

Rabbit anti human plasma protein sera and specific rabbit anti-human plasma protein sera against γ_{1M}, γ_{1A} 7S γ and α_{2M} globulins and lipoproteins were prepared by immunization of rabbits with human serum or purified serum fractions or were obtained from Behringwerke A G Marburg/L. Germany The specific anti sera were absorbed and the specificity was tested by immunoelectrophoresis and agar diffusion experiments against whole human serum and purified γ_{1M} γ_{1A} and 7S γ globulin preparations

Immunochemical determinations of γ_{1M} and 7S γ globulins were performed with a modified Boyden agglutination inhibition technique (16)

Serological Methods

Sensitized sheep blood cells Sensitizing serum from rabbits was used in a concentration containing one minimal agglutination dose (MAD) per ml of a 1 5 per cent sheep erythrocyte suspension

Sensitized sheep cell agglutination test (SCAT). Inactivated serum in 1 5 ml [illegible]

[illegible] inactivated serum (56° C, 30 min) or serum fractions were serially diluted twofold in [illegible] 8 2 with 0 15 M NaCl To each tube 1 0 [illegible]

[illegible]

[illegible] tions were analysed for antistrepto- [illegible] with the modification introduced by [illegible] *of titre* The titre is expressed in [illegible] units (IU) ml as compared with standard sera from the Department of Biol Stand of the State Serum Institute *Copenhagen*, Denmark

EXPERIMENTS AND RESULTS

Rheumatoid Factors

Selection of buffer for gel filtration Preliminary experiments with separation of rheumatoid factors (RF) on Sephadex G-200 in TRIS-NaCl buffer gave quantitative recovery of the activity tested by the acryl fixation test (AFT) but a substantial loss of the activity in the sheep erythrocyte agglutination test (SCAT) The effect of the presence of TRIS NaCl on these two activities was investigated by dialysing selected sera first against TRIS-NaCl buffer for 24 hours and subsequent-

The present investigation describes the purification of rheumatoid factors (RF) and antistreptolysins O (AST) from human sera using these techniques. A preliminary report has been published (19). Gel filtration has been performed on Sephadex G-200 and on pearl condensed agar gel. The latter gel permits separation of molecules of larger size than the former (2, 14, 22).

MATERIAL AND METHODS

Human sera with high titre of rheumatoid factor (RF) and/or high antistreptolysin O activity (AST) were selected. The sera were fractionated fresh or after storage at —20° C. In some experiments most of the lipoproteins were removed by preparative ultracentrifugation at 105 000 g at a density of 1.06 g/ml for 9–19 hours before separation (4).

Chemical Methods

Gel filtration on cross linked dextran gel (Sephadex G 200, AB Pharmacia, Uppsala, Sweden) 200–270 dry mesh (US sieve series) was performed at +3°–+8° C on plexiglass columns equipped with cooling jackets. The elution rate was usually 1.15 ml/cm²/hour. Further details about the technique have been published elsewhere (20).

Gel filtration on pearl condensed agar gel was performed on 3.5 per cent agar (Difco Noble Agar) condensed as described by *Bengtsson & Philipson* (2). The sieve fraction 100–140 wet mesh (US sieve series) was packed in plexiglass columns. The gel bed was 2.6 × 38 cm and was used at room temperature (about +22° C). The elution rate was kept at 1–1.5 ml/cm²/hour by hydrostatic pressure or a peristaltic pump.

Recycling gel filtration according to *Porath & Bennich* (36) was performed on agar columns as described in detail elsewhere (22).

Buffer solutions. In most of the experiments 0.05–0.1 M sodium or potassium phosphate buffers pH 7.0–7.4 containing 0.2–0.4 M NaCl and 0.02 per cent sodium azide was used (phosphate-NaCl buffer). In some experiments when AST was separated 0.1 M TRIS-HCl buffer pH 8.0 containing 0.5 M NaCl and 0.02 per cent sodium azide was used (TRIS NaCl buffer).

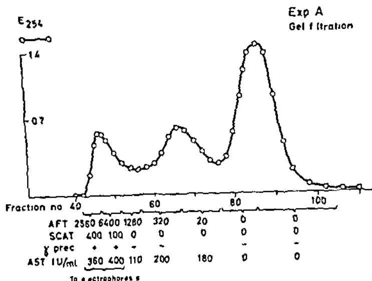
Preparative electrophoresis was performed either on granulated starch block of dimensions 50 × 30 × 1 cm according to *Kunkel* (24) using barbital buffer pH 8.6 ionic strength 0.1 and 400–440 V, 100–110 mA for 18–26 hours at +6° C or on a vertical column (4 × 81 cm) packed with cellulose according to *Porath* (33). The column was cooled to +5° C with a cooling jacket. The electrophoresis was run in 0.1 M TRIS–0.5 M glycine pH 8.1 at 465–710 V, 80–115 mA for 47–50 hours or in 0.5 M TRIS 0.021 M EDTA and 0.075 M boric acid pH 8.9, at 760 V, 60–65 mA for 44 hours.

Protein concentration of the eluate was continously registered as transmission at 254 mμ in a Uvicord absorptiometer (LKB Produkter, Stockholm, Sweden) or was determined in collected fractions by a modified Folin method (5). In the graphs protein concentration is plotted as optical density at 254 mμ or at 500 mμ for the Folin test.

Concentration of the fractions was made by ultrafiltration in collodium bags (Membranfilter A. G. Gottingen, Germany).

Immunoelectrophoresis was performed according to *Scheidegger* (38) with the following modifications. *Bacto agar (Difco) in 1.5 per cent solution in barbital HCl* buffer pH 8.2, ionic strength 0.05 was poured in 1 mm thick layers on 26 × 76 mm glass slides. The sample in 0.5–3 μl was separated by electrophoresis for 60–90 minutes at a potential gradient of 6 V/cm and the pattern developed with about 75 μl of antiserum.

Double diffusion in agar gel (30) was performed on glass slides covered with a 1 mm thick layer of the agar solution described above. A 1 or 2 mm × 65 mm through was cut in the middle for the antiserum and cylindrical wells (2 or 4 mm in diameter) were arranged in rows at 3 or 4 mm distance on either side for the



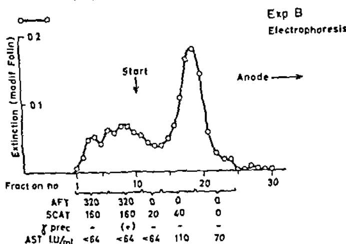


Fig 1

Exp A Gel filtration of 17 5 ml serum (Experiment F 79 in Table 2 and 3) on a

an l SCAT and < 36-64 IU ml for AST *Exp B* Separation of the macroglobulin fraction from the experiment in Fig 1 Exp A by electrophoresis on starch block Total protein in eluted fractions was determined by the Folin method RF and AST were determined as described in Fig 1 Exp A

separate macroglobulins of human serum (22) A human serum with high RF activity was fractionated on a Sephadex G 200 column The macroglobulin fraction contained most of the RF activity, α and γ_1-macroglobulins and large size lipoproteins It was concentrated and gel

ly against phosphate-NaCl buffer The original sera and the dialysates were tested for AFT and SCAT activity Table 1 shows that TRIS NaCl irreversibly reduces SCAT with 2–4 titre steps although the AFT activity is unimpaired Dialysis against phosphate-NaCl did not have this effect In the experiments with RF reported below only phosphate NaCl buffer was used

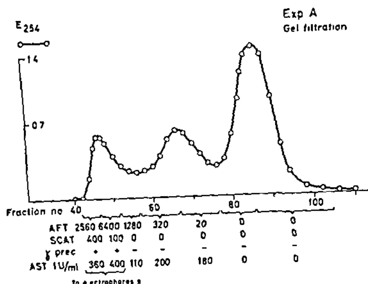
TABLE 1
Effect of TRIS Buffer on the Sensitized Sheep Cell Agglutination (SCAT) and the Acryl Fixation Tests (AFT)

Serum no	Titre					
	Original test		Dialysis against TRIS NaCl buffer		Redialysis against Phosphate NaCl buffer	
	AFT	SCAT	AFT	SCAT	AFT	SCAT
1	320	320	320	80	320	80
2	160	640	160	40	160	40
3	640	640	640	40	640	80
4	640	640	640	80	640	80

Separation on Sephadex G-200 and subsequent preparative electrophoresis Five human sera with rheumatoid factors in high titres were gel filtered on Sephadex G 200 The activity was recovered, as seen in Table 2, mainly in the fractions eluted in or close to the void volume, subsequently referred to as the macroglobulin fraction Fig 1 Exp A also shows, however, that some activity was found between the macroglobulin fraction and the second protein peak, and also in this peak, which contained the 7S γ-globulins In one experiment two maxima were observed (Exp no F 72, Table 2) with 9 per cent of the activity eluting close to the 7S γ globulin fraction These findings indicate that rheumatoid factors can be associated with proteins smaller than the usual γ_{1M} globulins

The macroglobulin fractions were pooled, concentrated and separated by granular starch block electrophoresis as shown in Fig 1 Exp B RF activities could be demonstrated in the γ β region The large peak contained α_2 macroglobulin The fractions with RF activity in Fig 1 Exp B were also tested by immunoelectrophoresis using anti-human serum and specific anti-γ_{1M}, anti γ_{1A} and anti-7S γ-globulin sera The concentrate from the slowest migrating fractions showed only one component which corresponded to γ_{1M} globulins The distribution and recovery of RF activities in all sera after gel filtration on Sephadex G-200 is given in Table 2 Recovery of serological activities over the Sephadex columns ranged from 17 98 per cent

Separation on agar gel Rheumatoid factors have been claimed to consist also of components with sedimentation coefficients larger than 19S (26) This initiated an attempt to separate rheumatoid factors of different sizes by gel filtration on pearl condensed agar which is able to



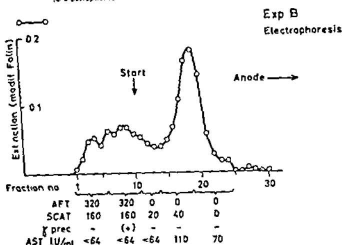


Fig 1

Exp A Gel filtration of 17.5 ml serum (Experiment F 79 in Table 2 and 3) on a Sephadex G 200 column 3 2 × 97 5 cm Elution buffer was 0 05 M potassium phosphate pH 7 0 with 0 2 M NaCl and fraction volume 5 2 ml Total protein in the eluate was determined as optical density at 254 mμ Rheumatoid factor (RF) was measured by SCAT AFT and γ precipitation tests and antistreptolysin (AST) was determined in pooled and concentrated fractions as indicated 0 means < 10–20 in titre of AFT and SCAT and < 36–64 IU ml for AST *Exp B* Separation of the macroglobulin fraction from the experiment in Fig 1 Exp A by electrophoresis on starch block Total protein in eluted fractions was determined by the Folin method RF and AST were determined as described in Fig 1 Exp A

separate macroglobulins of human serum (22) A human serum with high RF activity was fractionated on a Sephadex G 200 column The macroglobulin fraction contained most of the RF activity, α_2- and γ_1-macroglobulins and large size lipoproteins It was concentrated and gel

filtered on a 3 5 per cent agar gel As seen in Fig 2 the RF was recovered in a broad peak In a further attempt to resolve the RF activity of different molecular size the positive fractions were submitted to a new agar gel filtration using the recycling technique described by *Porath &*

TABLE 2

Separation of Rheumatoid Factors (RF) by Gel Filtration on Sephadex G 200

Exp no	Serum	Titre† serum SCAT	serum AFT	Macroglobulin fraction SCAT	Macroglobulin fraction AFT	Lower molecular size fractions SCAT	Lower molecular size fractions AFT	Total recovery %
E 47	F B	n t §	2560	n t §	1740	n t §	0*	68
E 58	E R		3200		1600		0	50
		160		42		0		26
E 72	S La		1600		500		0	31
		800		650		75		90
E 79	S La		3200		3040		100	98
		800		150		0		19
E 161	C W		12800		8530		n t §	67
		3200		530		n t §		17

§ n t = not tested * 0 = titre less than 10–20

† Titres corrected to correspond to original serum volume

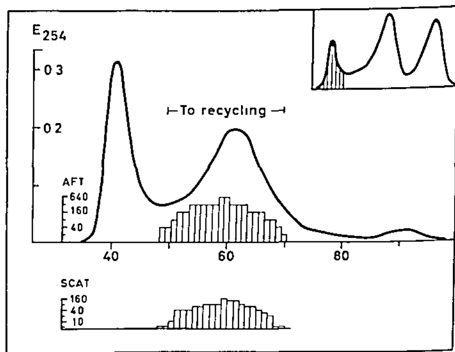


Fig 2

Gel filtration on pearl condensed 3 5 per cent agar of the macroglobulin fraction of 10 ml of serum obtained by gel filtration on Sephadex G 200 (inserted diagram) Elution buffer was 0 1 M sodium phosphate pH 7 4 containing 0 4 M NaCl and 0 02 per cent sodium azide Protein and RF activity was determined as described in Fig 1 Exp A

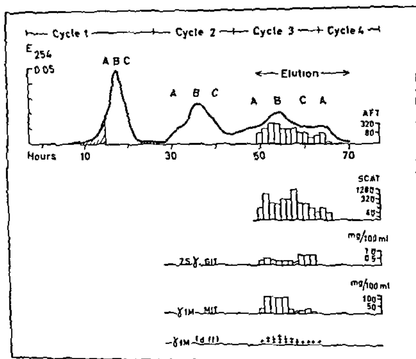


Fig 3

Recycling gel filtration on a 3 5 per cent agar gel column of pooled and concentrated fractions with RF activity from experiment in Fig 2 Shaded areas represent parts of the eluate which were *not recycled* The elution was started in cycle 3 when part of component A already had passed This part was eluted in cycle 4 partly mixed as in Fig 2 Protein and determined by diffusion a modified Boyden

Bennich (36) Fig 3 shows that the RF activity was then recovered in a broad range with a tendency for two maxima for the SCAT activity In the fractions 7S γ-globulins were determined by the use of a sensitive agglutination inhibition test, based on the technique of Boyden (GIT in Fig 3) (16) The front peak in the SCAT activity did not, however, coincide with a peak of 7S γ globulins

The tendency for separation of RF of different elution rate is also demonstrated in a similar experiment in Fig 4 The gel filtration column did not permit further cycles since the fastest fraction would in the next cycle have run into the slowest one

Antistreptolysins Gel filtration on Sephadex G 200 followed by preparative electrophoresis was performed on six sera with high titres of antistreptolysin O Two of these sera were also studied for RF activity Fig 1 shows a typical elution *diagram* from a Sephadex G 200 column of a serum with high AST and RF activity Most of the AST was re

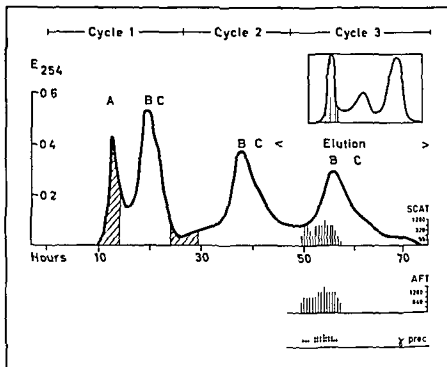


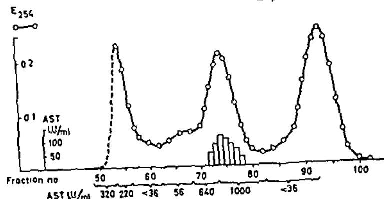
Fig 4

Recycling gel filtration on 3 5 per cent agar gel column of the macroglobulin fraction of 15 ml of serum obtained by gel filtration on Sephadex G-200 (inserted diagram) Elution buffer was the same as in Fig 2 Protein and RF was determined as described in Fig 1 Exp A

covered in the macroglobulin fraction although about one third of the activity was observed in the 7S γ-globulin region AST could be quantitatively recovered in the α_2-region when the combined and concentrated macroglobulin fraction was examined in preparative electrophoresis as shown in Fig 1 Exp B This region only contained α-β lipoproteins but no γ_{1M}, γ_{1A} or 7S γ-globulins when tested by immunoelectrophoresis A high activity in the macroglobulin fraction with similar electrophoretic behaviour was obtained in a second experiment (Fig 5 Exp A) The recovery of AST activity in the macroglobulin fraction after gel filtration ranged from 20–81 per cent of the total activity of the untreated sera (Table 3)

To further illustrate the lipoprotein nature of the AST activity in the macroglobulin fraction, the combined fractions with AST activity in the electrophoresis of Fig 1 Exp B were adjusted to a density of 1 06 g/ml with sodium chloride and centrifuged for 9 hours at 105 000 g The contents of the tube were then collected as 7 fractions from a hole in the bottom of the tube AST activity could then only be demonstrated in the top-fraction

These findings prompted gel filtration studies of the same serum (E P) before and after removal of lipoproteins Fig 5 Exp A shows that the untreated serum had about 20 per cent of the total AST activity



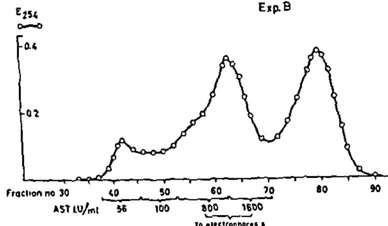


Fig 5

Gel filtration of serum E.P. (F 75 and F 119 of Table 3) before and after depletion of the lipoproteins *Exp A* 3.5 ml of serum was gel filtered on a 3.2 × 97.5 cm Sephadex G-200 column in 0.05 M potassium phosphate pH 7.0 containing 0.2 M NaCl and 0.02 [illegible]

[illegible]

Indicated Protein was analysed as described in Fig 1 Exp A

in the macroglobulin fraction but with most of the lipoproteins removed (Fig 5 Exp B) only about 1 per cent of the activity was found in this fraction No evidence of γ_{1M}-character of the AST activity was found in the sera investigated

AST activity eluting as 7S γ-globulins on Sephadex G-200 was also further purified by preparative electrophoresis As seen in Fig 6 all activity detected in concentrated fractions from the electrophoresis experiments was found in the γ region In immunoelectrophoresis using anti human serum and specific antisera only 7S γ and small amounts

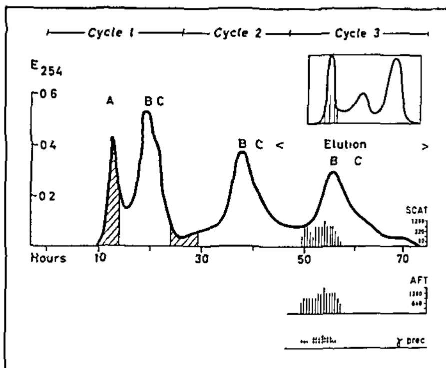


Fig 5

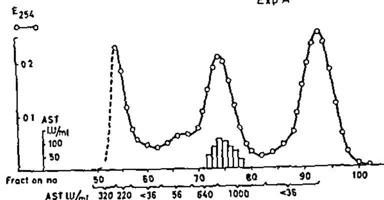
Recycling gel fi
of 15 ml of ser
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in Fig 1 Exp A

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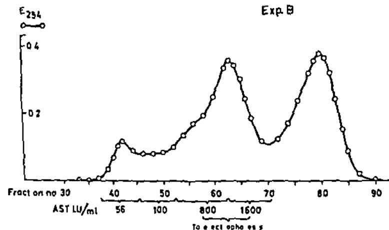


Fig 5

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AST activity eluting as 7S γ globulins on Sephadex G 200 was also further purified by preparative electrophoresis. As seen in Fig 6 all activity detected in concentrated fractions from the electrophoresis experiments was found in the γ region. In immunoelectrophoresis using anti human serum and specific antisera only 7S γ and small amounts

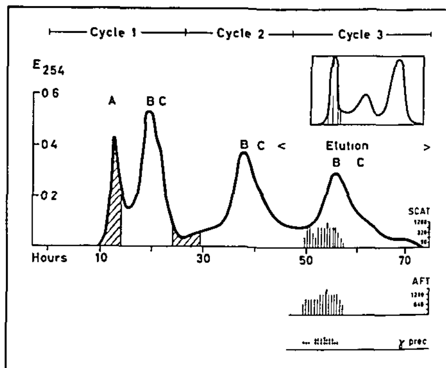


Fig 4

Recycling gel filtration on 3 5 per cent agar gel column of the macroglobulin fraction of 15 ml of serum obtained by gel filtration on Sephadex G 200 (inserted diagram) Elution buffer was the same as in Fig 2 Protein and RF was determined as described in Fig 1 Exp A

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These findings prompted gel filtration studies of the same serum (E P) before and after removal of lipoproteins Fig 5 Exp A shows that the untreated serum had about 20 per cent of the total AST activity

DISCUSSION

The present investigation reports the application of gel filtration on cross linked dextran (Sephadex G 200) and on pearl condensed agar to the separation and purification of rheumatoid factors (RF) and antistreptolysins (AST)

γ_1M globulins with RF activity were obtained in a highly purified state by gel filtration and preparative electrophoresis With the same techniques AST was recovered in the purified 7S γ globulins containing trace amounts of γ_A globulins Since γ_{1A} globulins elute between the macro globulin fraction and the center of the 7S γ globulin peak in gel filtration (8 16 21) and the AST activity coincides with the 7S γ globulin peak (Fig 5 Exp A) it is justified to assume that the AST activity is of 7S γ globulin type Thus RF was recovered in the γ_1M globulin preparation and AST in the γ globulin fractions in accordance with the findings of other investigators (1 11 26 31) concerning the type of immuno globulins associated with these antibody activities The results also illustrate the efficiency of the gel filtration electrophoresis technique for purification of immunoglobulins which has also been demonstrated for several other antibodies (10 16 19 21)

However some results deserve special attention It was first established that dialysis against TRIS buffer irreversibly inactivated the major part of the SCAT activity although the AFT activity of the rheumatoid factor was unimpaired

Secondly the results obtained by recycling gel filtration on agar gel demonstrated a tendency for two macroglobulin fractions with RF activity The separation was not complete but reproducible The broad elution pattern of the RF activity might possibly be due to adsorbtion effects on the agar gel *e g* to the sulphate groups of the agaropectin The agar used contained about 1 per cent sulphate as dry weight (13) No evidence for adsorbtion effects on agar has however been obtained when different macroglobulins were tested in this system (22 41) Furthermore buffers of high ionic strength were used throughout to minimize such effects as well as protein protein interactions With regard to the basic principle of gel filtration (28 34 35) it therefore appears justified to ascribe tentatively the two fractions different molecular sizes

The two components of RF did not contain significantly different amounts of 7S γ globulins but differences in the γ globulin precipitation test relative to the SCAT activity was demonstrated between the two peaks Larger amounts of material are however required to study these fractions in the analytical ultracentrifuge and to decide whether the larger RF consists of aggregates which previously has been described in sera with high RF activity (26)

A small quantity of RF activity was recovered later than the macro globulin fraction in gel filtration in 2 out of 4 sera Although inconclu

TABLE 3

Separation of Antistreptolysins (AST) by Gel Filtration on Sephadex G 200

Exp no	Serum	AST in serum IU ml	AST in eluate IU ml*			Total recovery %
			Macro globulin fraction	Inter mediate fraction	7S γ globulin fraction	
F 57	G E §	560	<90		500	89
F 72	S I ı	1000	356(36)		375(38)	74
F 79	S La	280	*226(81)*	*33(12)*	*113(40)*	133
F 75	I P	1800	356(20)		1120(62)	82
F 119	F P §	2500	22(1)	52(2)	1230(49)	52
F 109	G K §	1100	<64		1450	132
F 124	L Li §	800	56(7)		500(63)	70

* Activities corrected to correspond to original serum volume Number in brackets are per cent of total serum AST activity

§ Serum depleted of lipoprotein by ultracentrifugation

of γ_{1A}-globulins were detected in these fractions Similar results were obtained in two further experiments It therefore appears established that the AST activity found in this region was of antibody nature Recovery and initial titres of AST in the different gel filtration experiments have been summarized in Table 3 The total recovery of AST activity over Sephadex G-200 ranged from 52 133 per cent Between 20–89 per cent of applied activity was recovered after electrophoresis of the 7S-fractions

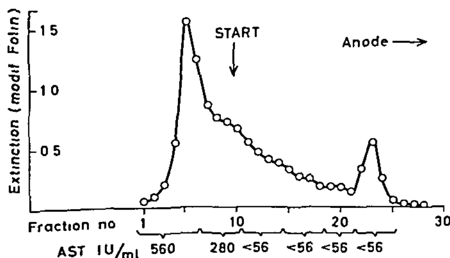


Fig 6

Preparative electrophoresis on starch block of 7S γ globulin fraction from the experiment in Fig 5 Exp B Protein and AST was determined as described in Fig 1 Exp B

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The AST activity associated with the 7S γ-globulins in gel filtration and zone electrophoresis is considered to be an immunoglobulin and thus of antibody nature The AST activity found in the macroglobulin fractions was shown to accompany the low density large molecular weight lipoproteins in zone electrophoresis and in the ultracentrifuge In three sera 20–81 per cent of the total activity was associated with the macroglobulin fraction When lipoprotein depleted sera were gel filtered, however, 1–7 per cent of the activity was recovered in this fraction This remaining activity may be due to lipoproteins, remaining after incomplete removal or to the occurrence of higher density lipoproteins with AST activity The possibility that this activity represents γ_{1M} globulins should not be overlooked although no evidence in this direction was obtained

Thus it appears that in the sera investigated AST antibody was confined to the 7S γ and/or γ_{1A}-globulins with no activity in the γ_{1M} globulins Since the sera had high titres of AST a dominant population of 7S γ-antibodies should be expected Antibodies of γ_{1M}-type might have been found if sera from early immunization stages were selected (6, 7, 29) but no such serum was available The AST activity in the macroglobulin fraction was obviously associated with lipoproteins Similar unspecific activity has previously been described by several investigators (1, 31) The high amount of unspecific AST in the sera investigated (20–81 per cent of the total activity) indicates that lipoprotein depletion ought to be included routinely prior to AST titrations (15)

SUMMARY

Rheumatoid factors and antistreptolysins have been separated and purified by gel filtration on cross-linked dextran gel (Sephadex G 200) and pearl condensed agar gel and subsequently by preparative electrophoresis

Rheumatoid factors showed a heterogeneous elution pattern suggesting two γ_{1M} globulin containing fractions, probably of different molecular sizes The presence of lower molecular size rheumatoid factor is discussed

TRIS-buffer irreversibly inactivates most of the activity measured by the sensitized sheep cell agglutination test

Antistreptolysins of antibody type was found in the 7S γ-globulin fraction and an unspecific antistreptolysin was bound to lipoproteins in the macroglobulin fraction No antistreptolysin was found to be of γ_{1M}-type

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STUDIES ON THE ANTIGENIC STRUCTURE OF *T. PALLIDUM* 5. ATTEMPTS TO ISOLATE POLYSACCHARIDE ANTIGEN FROM NICHOLS' PATHOGENIC STRAIN

By

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Received 11.xi.63

In a previous paper (1) preliminary experiments were reported which suggested that it should be possible to isolate a polysaccharide antigen from pathogenic treponemes using the extraction procedure with phenol-water described by *Westphal et al.* (12).

It appeared from these experiments that a considerably larger number than the 5×10^9 treponemes used at that time was needed for the studies to be accomplished. Furthermore, it was realized that modifications in the original *Westphal* method were necessary in order to obtain a quantitative yield of the polysaccharide found in the treponemes. Because of the well-known difficulty in securing a sufficient quantity of pathogenic treponemes, which only can be cultivated in rabbit testes, the cultivable and apathogenic *T. Reiter* was used as model in experiments dealing with the development of a suitable variant of the phenol-water method to be used on treponemes. These studies have resulted in a method for extracting polysaccharides from treponemes (2).

The encouraging serological experiments with the polysaccharide antigens from apathogenic treponemes (3, 4) have been a further stimulus to an investigation of the pathogenic strain of *T. pallidum*.

In the present study attempts have been made to increase the yield of treponemes and to rid the treponeme suspension of rabbit testis tissue. A material amounting to 800×10^9 treponemes has been submitted to extraction of polysaccharide employing the phenol-water method described earlier (2). Furthermore, extraction has been performed using both treponeme-infected and normal whole rabbit testes. The three extracts have been examined as to reactivity with syphilitic antibodies in complement fixation tests.

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3 g dry weight) was submitted to extraction of polysaccharide by the phenol-water method mentioned above. The yield of purified extract amounted to 17.5 mg (dry weight). The polysaccharide content of the extract was 31 per cent when 1 mg dry matter was dissolved in 1 ml water and subjected to the anthrone reaction. Nucleic acid was demonstrated in the extract from the pathogenic treponemes, as was the case with the polysaccharide antigens from *T. Reiter* & *T. Kazan* isolated by the same method.

In order to be used as antigen in a complement fixation test 1.5 mg of the extract was dissolved in 1 ml of water and diluted 1:10 and 1:100. No reaction was observed with control sera from patients with syphilis, not even when 10 mg of the extract was dissolved in 1 ml of water and used as antigen in the just mentioned dilutions (Table 1).

Extract from Non-Purified Suspension of Pathogenic Treponemes

The material to be extracted comprised about 10×10^9 treponemes in 3.5 g dry matter. It appears from Table 1 that the extract from the non-purified suspension reacts with the syphilitic control serum in the complement fixation test. This antigen also contained about 30 per cent polysaccharide and was thermostable (100°C for 30 minutes).

Extract from Rabbit Testes Infected by Pathogenic Treponemes

When extract from whole uncut and non-eluted rabbit testes infected by pathogenic treponemes was subjected to the serological examination as to antigenic properties, a reaction was observed again with syphilitic serum (Tables 1). The antigen was thermostable and contained polysaccharide of the same order of magnitude as before.

Extract from Normal Rabbit Testes

Applying this extract as an antigen against syphilitic serum revealed the reactivity shown in Table 1. This antigen contained also about 30 per cent polysaccharide and was thermostable.

TABLE 1

Results of Complement Fixation Test Using Various Antigen Preparations Against Syphilitic Serum

Antigen	Complement fixation test
Cardiolipin	12
Extract from purified treponemal suspension	negative
Extract of non-purified treponemal suspension	7
[illegible]	7
	12

the number of treponemes in the testicular tissue This reatment has been tried but without satisfying results Consequently, in order to increase the yield attention has been focused on on a elution technique for the isolation of treponemes from the testes It is obvious that the number of treponemes which can be liberated from the testes will depend on the area of the cut surface In principle the more the sections the more the treponemes, but this also means more testis tissue debris which is rather difficult to remove Therefore, it is necessary to combine the elution technique with suitable differential centrifugation Several experiments have led to the following method The infected testis, which has been removed aseptically, is cut into two pieces by a section placed on the front side from the upper to the lower pole The section leaves a depth of about 2 mm tissue uncut Thus the testis can be opened like a book and sections can be placed as close as possible in the two main pieces The distance between the incisions is about 2 mm Afterwards the tissue is eluted in saline (2 ml per testis) for 20 minutes in a shaking machine which gives about 280 oscillations per minute with 4 cm stroke The suspension of treponemes and testis debris is then decanted The suspension is centrifuged (3000 G for 5 minutes) to remove the coarser debris The testicular tissue is eluted once more and the new suspension decanted and centrifuged as before The combined supernatants are submitted to 35 000 G for 20 minutes The supernatant is discarded and the sediment lyophilized Besides the treponemes the lyophilized sediment contains a considerable amount of testis debris which has remained suspended after the first centrifugation

Extraction of Lyophilized Material

The lyophilized treponemes are extracted with ether and acetone for one hour in a Soxhlet apparatus, and the residue treated with phenol and water (equal parts) in a Braun mixer for one hour at +4° C Centrifugation of the mixture separates a water phase (mainly containing nucleic acid), a phenol phase (containing protein), and an undissolved boundary layer between these phases The boundary layer is extracted with water in the mixer for one hour After centrifugation a second water phase is obtained which is submitted to dialysis to remove phenol The dialysed second water phase is evaporated in vacuo and after addition of methanol till a concentration of 75 per cent the polysaccharide is precipitated The precipitate is sedimented by centrifugation washed with methanol and acetone, and lyophilized

Extraction of Whole Rabbit Testes

The extractions of infected and normal testes are also carried out according to the above-mentioned method except that the lipoid extraction with ether and acetone is performed on the lyophilized final product because the testes are used in a whole and fresh condition Six to eight testes have been used for each experiment

Purification of the Extracts

The lyophilized extract is dissolved in water and the solution heated on a water bath (100° C for 30 minutes) followed by centrifugation (12 000 G for 20 minutes) By this procedure undissolved, suspended particles (mainly containing protein) are removed from the solution Experiments with the polysaccharide antigens from apathogenic treponemes have shown that this final purification does not decrease the reactivity of the antigens (5)

The various extracts have been examined in the complement fixation test described previously (1) against sera from patients with lues Furthermore examination has been performed on serum absorbed with cardiolipin antigen as described below

Absorption technique 6 ml cardiolipin antigen is concentrated in vacuo to about 1 ml after which 2 ml of 0 9 per cent saline is added Then 1 ml of this mixture 1 ml serum and 100 mg terra silicia are shaken in the above mentioned shaking machine for two hours at room temperature and centrifuged at 9000 G for 20 minutes at +4° C

RESULTS

Extract from Purified Suspension of Pathogenic Treponemes

A lyophilized material containing about 800 × 10⁹ treponemes (about

TABLE 2 (cont)

Serum no	Clinical diagnosis	Serological examinations			
		Quantitative CWRM KR MB	TPI	Qualitative	
				Cardio-lipin	Normal testis extract
34	Lues lat	2 ±	+	20	90
35	Lues lat	7 6 ++	+	0	20
67	Lues lat	8 7 +	+	0	0
69	Lues lat	8 6 ±	+	0	0
73	Lues lat	11 9 ++	+	0	80
27	LFD†	1/1 +	⋖	40	30
29	LFD obs	–/– +	—	100	40
75	Lepra	12 unr —	—	0	20
78	No lues	8 – +	—	0	40

The immediate conclusions to be drawn from the results of the serological experiments with the four types of extract is that the reacting substance derives from the testis tissue and not from the treponemes

The reactivity of the extract from normal testes has been further studied as shown in Table 2 The material includes sera from 51 patients with syphilis in various stages of this disease (treated and untreated) and from 4 patients with so-called "biological false positive" reactions The testis antigen was used in a dilution of 1:10 of a 1 per cent stock solution in the complement fixation test described earlier (1) Reactions with a percentage of haemolysis below 60 are called positive It will be seen that 40 of the 51 syphilitic sera were positive with the testis antigen The reactions with the 4 "biological false positive" sera were also positive, though weak In addition, the results of the Treponema Pallidum Immobilization Test (TPI) and the standard tests for syphilis (*Wassermann, Kahn, and Meinicke's* reactions) are given The results in Table 2 suggest that the testis substance reacts with the antilipoidal antibodies rather than with the immobilizing antibodies

TABLE 3

Syphilitic Serum Examined Before and After Absorption with Cardiolipin Antigen

	TPI	Cardiolipin antigen	Extract from normal testes
Before absorption	positive	8*	6
After absorption	positive	negative	negative

* Degrees of strength

Absorption Experiments

In order to see whether or not the extract from normal testes reacts with antilipoidal antibody, a syphilitic serum was absorbed with cardio-

TABLE 2

Examination of Sera from 51 Patients with Lues in Various Stages and 4 Patients with 'Biological False Positive' Reactions

Serum no	Clinical diagnosis	Serological examinations			
		Quantitative *CWRM/KRMR	TPI	Qualitative §	
				Cardiolipin	Normal testis extract
18	Lues I u	8/5 +	—	0	0
25	Lues I t	12/6 ++	±	0	0
31	Lues I u	13/9 ++	±	0	20
33	Lues I t	-/- —	—	100	80
36	Lues I u	2/2 +	—	20	90
37	Lues I t	8/3 +	+	0	0
38	Lues I t	5/3 ±	±	0	0
40	Lues I u	4/2 +	×	10	0
41	Lues I t	8/6 ++	—	0	0
42	Lues I t	8/5 ±	—	0	0
52	Lues I t	13/9 ++	—	0	0
53	Lues I u	7/6 ++	—	0	60
54	Lues I t	8/6 ±	+	0	0
58	Lues I t	7/3 +	+	0	0
59	Lues I t	11/9 ++	—	0	40
68	Lues I u	/- —	—	100	40
76	Lues I t	6/3 ±	×	0	20
77	Lues I t	/4 —	—	100	60
16	Lues II t	12/5 ±	+	0	0
20	Lues II u	19/12++	+	0	0
24	Lues II u	11/8 +	+	0	0
26	Lues II u	15/9 ++	+	0	0
30	Lues II t	/- —	+	100	60
32	Lues II t	14/9 ++	+	0	0
44	Lues II u	12/6 ++	×	0	0
45	Lues II t	11/9 ++	+	0	0
46	Lues II t	11/7 +	×	0	0
47	Lues II t	11/10++	×	0	0
48	Lues II u	11/9 ++	±	0	0
49	Lues II t	11/6 ++	+	0	0
50	Lues II t	11/9 +	+	0	0
51	Lues II t	12/7 +	+	0	0
55	Lues II t	8/6 ++	±	0	0
57	Lues II t	4/2 —	—	10	90
62	Lues II t	6/1 —	—	0	60
63	Lues II t	1/1 —	—	40	90
65	Lues II t	5/3 ±	—	0	10
66	Lues II t	12/9 ++	+	0	0
72	Lues II t	5/3 —	+	0	40
74	Lues II u	13/9 ++	+	0	0
82	Lues II t	11/7 ++	×	0	0
83	Lues II u	10/6 ++	+	0	20
84	Lues II t	11/8 ++	×	0	20
86	Lues II t	10/6 +	+	0	0
8	Lues lat	4/2 ±	+	10	10
11	Lues lat	-/ —	×	100	90

* CWRM/KR in degrees of strength
§ Percentage haemolysis
† Lupus erythematosus disseminatus
u = untreated t = treated — = negative × = not performed unr = unreadable

TABLE 2 (cont)

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* Degrees of strength

Absorption Experiments

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lipin antigen The results are shown in Table 3 It will be seen that the reactivity disappeared after absorption with cardiolipin antigen, indicating that the testis antigen actually does react with the antilipoidal antibodies

DISCUSSION

The failing reactivity with syphilitic serum of the extract from the purified suspension of 800×10^9 pathogenic treponemes (3 g dry matter) is contradictory to the prelimenary findings, when a thermostable substance containing polysaccharide was isolated from only 5×10^9 treponemes (1) However, the prelimenary extractions were carried out on a material which was less purified and thus contained a considerably larger amount of testicular tissue in proportion to the content of treponemes

Repetition of the extraction on a non purified material of treponemes (10×10^9 treponemes in 3 5 g dry matter) led to the isolation of a substance reacting with syphilitic serum and containing polysaccharide of the same order of magnitude (*viz* 30 per cent) as the above-mentioned non reactive substance

The preliminary experiments (1) were thus reproduced and it seems reasonable to conclude that the failing reactivity of the extract from the purified suspension is due to removal of the antigenic substance during the differential centrifugation employed for the purification of the treponemal suspension

In order to determine whether the reacting substance in the non-purified material derives from the testes of from the treponemes extraction was carried out also on normal non-infected rabbit testes The results showed that the extract of normal testes possessed the same properties as the extracts of the non-purified treponemal suspension and treponemal infected testes, *viz* a reactivity with syphilitic sera (Table 1 and 2) and a content of polysaccharide about 30 per cent

The question is now whether the reactivity with syphilitic sera (Table 1) is due to the polysaccharide This theory seems to be rather improbable for several reasons Firstly, about 30 per cent polysaccharide has been demonstrated in all of the extracts including the non-reacting one from the purified treponemes As it will appear from the calculation below, most of the substance in the purified treponemal suspension originates from the testicular tissue Thus, the demonstrated polysaccharide in this fraction also derives from the testis tissue There can be no doubt that the polysaccharide in the remaining three (reactive) extracts derives from testis tissue because of the predominating content of this in the initial material There is no reason to believe that the same polyscecharide should be non reactive in one extract while reactive in the other three (compare Table 1)

Secondly, it has been shown by the absorption experiments that the

testis antigen reacts with antilipoidal antibody This reactivity can hardly be due to polysaccharide

Thirdly, the usual high specificity of polysaccharide from micro-organisms must be borne in mind (1, 2) It is thus hard to believe that testis polysaccharide should give any reaction with syphilitic antibodies

The failing reactivity of the extract from the purified treponemal suspension, which has contained 800 × 10⁹ treponemes, arises the question whether this material is too small for the extraction of polysaccharide applying the phenol water method

In this connection, *Gelperin's* study from 1949 (7) must be borne in mind *Gelperin* has shown that 10^9 *T Reiter* contains 0 8 mg of dry matter This fact used in the conservative estimate given below supports the suggestion that the material is too small It is assumed that *T pallidum* (Nichols' pathogenic strain) has an average dimension of 15 × 0 18 microns, and that the size of *T Reiter* is twice that of *T pallidum, viz* 30 × 0 36 microns (11) Furthermore, if the treponemes are regarded as cylinders, the volume ratio $\frac{T\ Reiter}{T\ pallidum} = 8$ According to *Siefert* (10) the content of dry matter of these treponemes is of the same order of magnitude Thus, in analogy with *Gelperin*, the dry weight of 10^9 *T pallidum* is about 0 1 mg, and the material comprising about 800 × 10^9 treponemes has only contained about 80 mg treponemal dry matter Taking these remarks concerning the proportions between the dimensions of *T Reiter* and *T pallidum* into account, 80 mg must certainly be considered a maximum value It must be emphasized here that the above-mentioned proportions between *T pallidum* and *T Reiter* are selected with caution in order to make the calculation safe In reality, the average *T Reiter* is considerably larger than *T pallidum*

Experiences from the extraction of polysaccharide from apathogenic treponemes have shown that it is preferable to have at least 1 g of dry weight of the initial material in order to get a reasonable yield for serological examination This is at least the case when the phenol water method (2) is used for extraction

The maximum 80 mg of treponemes was obtained from about 550 rabbits In order to get a sufficient material, at least 6500 rabbits would be necessary as long as it is not possible to cultivate *T pallidum in vitro*

The above mentioned 80 mg treponemal dry matter was contaminated with about 3000 mg testis debris which could not be removed by differential centrifugation Therefore, when *Siefert* (10) describes the content of 15 amino acids found in *T pallidum* isolated from testes, without mentioning the content of amino acids in the testis tissue, it is doubtful whether these amino acids actually are derived from the treponemes only and not from the testes

The isolated testis antigen which reacted with syphilitic sera and,

to a minor extent, with so called "biological false positive" sera, is thermostable. The absorption experiments demonstrated that the testis antigen reacted with antilipoidal antibodies. In 1962 *McLeod* (8, 9) demonstrated a thermostable antigenic substance in the TPCF antigen which reacted with antilipoid antibodies (reagin). This substance may be similar to the above-mentioned testis antigen. *McLeod* states that "TPCF is a complex of reacting substances, contaminated with a substance reacting with reagin." The complexity of the TPCF antigen was to be expected, in the first place on account of the isolation procedure and secondly because of the overwhelming amount of testis tissue which is found even in a purified suspension of treponemes isolated from testes. These considerations and facts give occasion for doubting whether any of the reacting substances in the TPCF antigen comes from the treponemes, since the content of these is so low in relation to the content of testicular tissue in the current eluate of rabbit testes.

Finally, the present study indicates that it is extremely difficult to obtain a sufficient material of pathogenic treponemes using the methods now available.

Renewed efforts should therefore be made to cultivate *T. pallidum* in an artificial medium. A thorough investigation of the apathogenic strains as regards taxonomy and growth requirements might be of value in this respect.

SUMMARY

The present study shows that the antigen of supposed polysaccharide nature previously obtained from *T. pallidum* (Nichols' pathogenic strain) comes from the testis tissue and not from the treponemes. Absorption experiments prove that this testis antigen reacts with antilipoidal antibodies.

Furthermore, it is found that the quantity of treponemes necessary to extract polysaccharide antigen is so large that the current methods for cultivating and isolating pathogenic treponemes seem *to be inadequate*. Renewed attempts should therefore be made to cultivate pathogenic treponemes in an artificial medium.

Finally, it is shown that the content of contaminating testis tissue in the treponemal suspensions is so dominating that the interpretation of biochemical studies is rendered difficult when the pathogenic treponemes have been isolated in the usual manner.

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Acta path et microbiol scandinav *61*, 150–151, 1964

BRIEF REPORTS

A METHOD FOR EXACT INTRACELLULAR LOCALIZATION OF TRITIATED SEX HORMONES BY AUTORADIOGRAPHY

By *Arne Attramadal*[1]

Little is known about the primary site of action of the steroid sex hormones and the ensuing chain of alterations responsible for the physiological effect. A better understanding of the mechanisms underlying the biological action of these hormones might be gained by determining the nature of the target cells and the precise site of action within the target cell. Tritiated sex hormones may provide a profitable tool for examination of these problems. There are however only few reports where autoradiography has been used for this purpose. So far the attempts have been
& *Jacobson* (1) this may be due to a heavy loss
ion and processing of the tissue.
carried out to estimate the loss of radioactive material by the use of routine fixation and processing procedures. For this purpose uterine tissue of female rats, given 50 µC oestradiol 17β 6,7 H^3 with a specific activity of 150 mC/mg (New England Nuclear Corp.), was used. The tissue was fixed in 4 per cent aqueous formaldehyde and 1 per cent aqueous osmium tetroxide respectively, both buffered to pH 7.4. Aliquots of the fixatives and of each medium involved in the processing were transferred to counting vials and dried under vacuum. All samples were digested in 0.5 ml of 1.0 M methanolic Hyamine hydroxide (Packard Istrument Co.). The digests were dissolved in 10 ml of A.R. toluene containing 0.5 per cent PPO (2,5 diphenyloxazole) and 0.03 per cent POPOP (1,4 bis 2 (5 phenyl oxazolyl) benzene) (Packard Instrument Co.—scintillation grade). The radioactivity was measured in a Tri Carb Spectrometer. In order to exclude recording errors each step in the registration method was controlled. All counts recorded were corrected for background activity, quenching and machine efficiency.

As shown in the table, 70–80 per cent of the total radioactive material present was lost by this method. Thus the conventional fixation and processing procedures are entirely unreliable when the aim is to obtain quantitative retention and to determine the exact localization of tritiated sex hormones. Special precautions are then necessary in order 1) to ensure a minimum loss of radioactive material during fixation and processing and 2) to retain the labelled hormone as exactly as possible at its original site of action.

Loss of Radioactive Material from Tissue during Fixation, Embedding and Staining

	Aqueous fixatives		Freeze drying + vapour fixation	
Radioactive substance	Oestradiol H^3	Oestradiol H^3	Oestradiol H^3	Progesterone H^3
Fixative	4 % formaldehyde pH 7.4	1 % osm tetrox pH 7.4	Osm tetrox vapour	Osm tetrox vapour
Loss in fixative (%)	20.55	3.86		
Loss in alcohol xylene (%)	58.04	72.76		
Loss in methacrylate (%)			0.09	0.11
Loss in PAS staining media (%)			4.35	4.43
Total loss (%)	78.59	76.62	4.44	4.54

A method has been developed to meet these requirements. One group of female rats was given 50 µC tritium labelled oestradiol with a specific activity of 150

Received 16.iii.64 *from Universitetet i Oslo, Institutt for Patologisk Anatomi, Rikshospitalet, Oslo* (Head: Professor Olav Torgersen, M.D.)

1 Fellow of The Norwegian Cancer Society (Landsforeningen mot kreft, Oslo)

mC/mg, while a second group received 50 µC tritium labelled progesterone with a specific activity of 118 mC/mM. All animals were sacrificed two hours after the injection. Small pieces of liver tissue not exceeding 0.5 × 1 mm in one of the dimensions were rapidly frozen in isopentane precooled in liquid nitrogen, frozen dried, fixed in vapour of osmium tetroxide and embedded in methacrylate in vacuo. Polymerization of the methacrylate was achieved by ultraviolet light (2), care being taken to avoid rise of temperature above —10° C. Alternating series of sections, one micron thick, were either left unstained or stained by the PAS method and subsequently prepared for autoradiography by the stripping film technique. The radioactive material lost during methacrylate embedding and in the different media involved in the PAS method was measured by liquid scintillation counting. The results are shown in the table. Thus by preparing autoradiograms of unstained tissue sections it is possible to keep the loss of radioactive material restricted to about 0.1 per cent. When, however, PAS staining is performed before the application of the stripping film the loss is about 5 per cent.

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Acta path. et microbiol. scandinav. *61*, 151–152, 1964

THE UPTAKE AND INTRACELLULAR LOCALIZATION OF OESTRADIOL 17β 6 7 H3 IN THE ANTERIOR PITUITARY AND THE HYPOTHALAMUS OF THE RAT

By *Arne Attramadal*[1]

Considerable *discrepancy exists* as to the mechanisms involved in the control of gonadotrophin secretion. *Increasing experimental evidence*, however, supports the concept that the *steroid sex hormones* exert a regulating effect on gonadotrophin secretion by acting as well upon target cells in the anterior pituitary gland as upon ... he nature nor the location of

... the sex hormones act exclu... cells either in the anterior pituitary or in the hypothalamic nuclei or in both places. A further *aim was to study* the particular cell types and the cell structures involved.

It is generally accepted that sex *hormones have* to be administered in amounts *within* physiological limits if information is wanted concerning their biological activities. Therefore tritiated sex hormon...

[1] ... Cancer Society (Landsforeningen mot kreft, Oslo)

xide (Packard Instrument Co) at 60° C The digests were dissolved in 10 ml of A R toluene containing 0 5 per cent PPO (2 5 diphenyloxazole) and 0 03 per cent POPOP (1 4 bis-2-(5 phenyloxazolyl) benzene) (Packard Instrument Co—scintillation grade) The radioactivity was measured in a Tri Carb Spectrometer All counts were corrected for background activity, quenching and machine efficiency The second group of animals was examined by autoradiographs with regard to the intracellular localization of the radioactive material and was given a single intraperitoneal injection of 75 µC tritium labelled oestradiol All animals were sacrificed two hours after the injection Fixation and processing of the tissue for autoradiographs were made according to the method previously recorded by the author (1)

The preliminary results of these studies show a significant selective accumulation of radioactive material in the anterior pituitary in amounts 5 8 times higher than the values registered in the blood plasma and 15 20 times higher than in the brain cortex and muscle tissue In the hypothalamic region radioactive material was recorded in amounts twice the concentration in the brain cortex and muscle tissue but somewhat lower than in the blood plasma Autoradiography of material obtained one hour after the injection revealed significant labelling of nerve cells in the supraoptic and paraventricular nuclei of the hypothalamus as well as of basophil cells in the anterior pituitary gland Moreover, in the supraoptic and paraventricular nuclei the labelling was almost exclusively localized to the nuclei of the nerve cells whereas the labelling of the basophils of the anterior pituitary was almost entirely limited to the cytoplasm This may indicate that the oestradiol acts upon the basophil cells of the anterior pituitary gland in a way different from that of the nerve cells of the hypothalamic nuclei Further experiments are in progress

These investigations have been aided by grants from The Norwegian Cancer Society and Nordisk Insulinfond

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ARIAS STELLA S PHENOMENON

By *Thorbjorn Berge*

In 1954 *Arias Stella* reported certain changes occurring in the endometrial glands during pregnancy (1) These alterations consist of the appearance of varying sized scattered or clustered hyperchromatic cells bulging into the glandular lumina Such changes had been described as early as 1933 by *Declman* who had however ascribed them to endometritis after abortion (3)

It is now generally agreed that such changes are due to the presence of chorionic tissue and that they are therefore of diagnostic interest It is however, not known how soon after conception the phenomenon may appear (2)

We observed such changes in a woman who had been pregnant for at most 14 days

A 26 year old divorced woman with regular menstrual cycles (5/27 days) sought advice for vaginal discharge Clinical examination revealed erosion of the posterior portio vaginalis Examination of the endometrial scrapings obtained on the 26th day of the cycle showed the characteristics of the secretory phase and changes of the type described by *Arias Stella* (Fig 1) There were no signs of inflammation One of the tissue fragments contained a fertilized ovum of normal histological appearance (Fig *2*) No contact between the ovum and the mucosal surface could be detected

Comment Judging from the regular menstrual time table of the patient the reported time of coitus and the histological appearance of the ovum the woman had been pregnant for at most 14 days

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Received 27 ii 64 *from the Department of Pathology Malmo General Hospital University of Lund Sweden*



Fig 1
Scattered and clustered cells with hyperchromatic nuclei bulging into the glandular lumen (Haematoxylin eosin × 200)



Fig 2
The 14 d egg (Haematoxylin eosin × 105)

The assumption that the phenomenon can be due to endometritis is not generally accepted and there is no reason to suspect such a causal factor in the present case as the scrapings showed no signs of inflammation Nor is there any reason to ascribe the changes to degeneration for the phenomenon has never been known to occur in the advanced secretory phase in the absence of pregnancy

We have however observed such changes, though less pronounced in 2 patients using contraceptive drugs (Anovlar)

Summary Report of a case in which focal endometrial changes of the type described by *Arias Stella* were demonstrated within at most 14 days of conception

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METHICILLIN-RESISTANCE IN *STAPHYLOCOCCUS AUREUS* APPARENTLY DEVELOPED DURING TREATMENT WITH METHICILLIN

By *Knud Riewerts Eriksen*

Penicillinase producing strains of *St*
methicillin containing media be made
stable and not associated with loss of
of virulent strains Several authors have expressed fears that such strains would appear as a result of methicillin therapy However methicillin resistant strains hitherto isolated from clinical cases have usually been found to occur without any *relationship to treatment with methicillin and are generally considered to be* "naturally resistant" The great majority of such strains have belonged to phage group III and have been resistant to penicillin streptomycin and tetracyclines many also to erythromycin and/or chloramphenicol (1, 3 5)

No case has been reported in which methicillin resistance has developed in an originally sensitive strain during treatment with methicillin

The purpose of this paper is to report the isolation of a strain of *Staphylococcus aureus* which appears to have developed a low-degree resistance to methicillin during treatment with this drug

TABLE 1

Susceptibility to Methicillin of Two Strains of Staphylococcus aureus Isolated before and after Methicillin Treatment

	Minimal inhibitory concentrations of methicillin in µg ml	
	Large inoculum (0 1 ml undiluted broth culture)	Small inoculum (0 1 ml of broth culture diluted to 10^{-6})
Strain isolated before treatment	2 5	1 25
Strain isolated after treatment	10	5

The patient was a man of 45 years with a pleural empyema from which a strain of *Staphylococcus aureus* was isolated It was resistant to penicillin and streptomycin and sensitive to methicillin The phage type was 52/52A 80 After treatment *with methicillin 6 grammes daily for 4 weeks staphylococci were still present* and a strain was now isolated which with the disc method commonly used in this laboratory (2) showed an inhibition zone only 30 mm in diameter as compared to at least 40 mm for methicillin sensitive strains Also this strain was resistant to penicillin and streptomycin and of phage type 52/52A/80

Susceptibility *to methicillin of the two strains* was estimated *in fluid and* solid media using both large and small inocula (see table) A four fold difference in

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sensitivity was observed No difference in sensitivity to oxacillin was found Both strains were potent penicillinase producers growing with a large inoculum in 10 000 µg/ml of penicillin G

The degree of methicillin resistance observed in the strain reported here was admittedly very low However no strain with such a high resistance has been found before in this laboratory except for methicillin resistant strains of the well known naturally occurring type

The resistance was of approximately the same order as that observed by *Stewart* (4) in some strains of *Staphylococcus albus* which apparently developed resistance during treatment

The re[illegible] hicillin [illegible] showed [illegible] sented a [illegible] ype and sensitivity pattern it was different from "naturally resistant" strains

No conclusions concerning the effect of methicillin treatment against the resistant strain could be drawn from the patients history a gram negative bacillus (hitherto unidentified) with rather pronounced methicillin inactivating properties also being present in the pleural fluid

Perhaps the presence of this strain was a contributing factor in the development of resistance As a result of inactivation the concentration of methicillin in the pleural fluid may very well have been kept at a level sufficiently low for allowing resistance to develop

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TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting November 30, 1963

H A Hansson & P Sourander STUDIES ON CELL DIFFERENTIATION IN CULTURES OF RETINA FROM RATS

B Thorell & F Ambs MICROSPECTROGRAPHIC ANALYSIS OF THE FUNCTIONAL HETEROGENEITY OF THE BLOOD LYMPHOCYTES

J Ericsson STUDIES ON THE ULTRASTRUCTURAL LOCALIZATION OF ACID PHOSPHATASES IN RENAL PROXIMAL TUBULAR CELLS WITH SPECIAL REFERENCE TO THE RELATIONSHIP BETWEEN ENZYME ACTIVITY AND PROTEIN ABSORPTION DROPLETS

Small pieces of renal cortex from young male Sprague Dawley rats were fixed in 6 25 per cent buffered glutaraldehyde for 3 4 hours and washed in 0 1 M cacodylate buffer for 1 7 days Frozen sections approximately 50 μ thick were incubated 10–15 minutes in the Gomori substrate for the demonstration of acid phosphatase and were then washed and postfixed in buffered osmium tetroxide After 2 hours osmium fixation the sections were rapidly dehydrated and embedded in Epon Electron microscopic investigations revealed a distinct localization of the reaction product (lead phosphate) in two types of cytoplasmic organelles in the cells of the proximal convoluted tubules *viz* the *cytosomes* and the *cytosegresomes* These organelles thus appear to contain acid phosphatase and by analogy with the biochemical evidence probably other acid hydrolases (lysosomal enzymes)

Hemoglobinuria was induced in the rats by the intravenous injection of lyophilized homologous hemoglobin and the relationship between hemoglobin absorption droplets on the one hand and the cytosomes and cytosegresomes on the other was studied by electron microscopy following the *in vivo* fixation of the proximal convoluted tubules with buffered osmiumtetroxide

The hemoglobin was absorbed from the tubular lumina by way of micropinocytosis The pinocytosis vacuoles containing hemoglobin increased in size by fusion with other hemoglobin filled vacuoles and moved toward the cytosomes and cytosegresomes which contained hemoglobin like material 4 hours following the injection At 12 16 hours many of the cytosomes contained ferritin like granules

These findings indicate that the intracellular digestion of hemoglobin occurs in the cytosomes and cytosegresomes and supply indirect evidence for the assumption that these organelles in addition to acid phosphatase contain cathepsin

Y Olsson P Sourander & L Svennerholm THE EFFECT OF INTRACEREBRAL INJECTION OF SFINGOLIPIDS IN RATS

G Nathorst Windahl & B Hellman LIPO HYALINE GLOMERULAR CHANGES IN MICE WITH HEREDITARY DIABETES

L Grimelius A MODIFIED SILVER PROTEIN METHOD TO DEMONSTRATE THE ARGYROPHIL CELLS OF THE ISLETS OF LANGERHANS

A modification of the silver protein method of Bodian Hamperl has been developed to differentiate the cells of the islets of Langerhans in ordinary laboratory animals As the method is affected by postmortem tissue changes the impregnation of human autopsy material will often be less satisfactory

The method differs from that of Bodian Hamperl as to the time of impregnation the concentration of the silver protein and the temperature (48 hours 2 g% and 55° C.) Metallic copper does not affect the impregnation *At present the silver* protein (from Degussa Company Frankfurt a M) is not commercially available

In the normal rat all the cells that are identified as *a* cells with Gomori s granule stains are impregnated with this method This has been demonstrated by restaining the impregnated sections with Gomori s paraldehyde fuchsin stain and by differential count of the islet cells

Restaining of sections from horse pancreas gives the same result There are silver positive islet cells in all the examined animals (calf cat, duck, dog guinea pig hamster monkey mouse pig rabbit sheep and bone fish (cottus scorpius)) and the argyrophil cells seem to agree with the a cells stained by Gomori s granule stains

J Thorell THE INFLUENCE OF INSULIN ANTIBODIES ON THE PLACENTAL TRANSFER OF INSULIN

Placenta is generally regarded as impermeable to insulin even if *divergent results* have been published The influence of the passage of insulin antibodies from mother *to fetus in insulin* treated mothers on the passage of insulin has been studied

Insulin immunized pregnant guinea pigs near term were injected with 2 mL insulin I^{131} *intravenously* The fetus were extracted via an abdominal *incision at various* intervals up to 20 hours after the injection and their plasma *and* that of the mothers were assayed for total activity and the insulin was identified by paper electrophoresis

No intact insulin was discovered in fetal plasma up to 3 hours after the injection but at longer *intervals an* increasing amount of γ globulin bound activity appeared This probably represents antibody bound intact insulin I^{131} The highest activity, 10-15 per cent of the *concentrations found* in the mothers plasma was found 20 hours after the injection

No antibody bound insulin was *found in the* amniotic fluid or in the stomach contents of the fetus No transfer was found in *non immunized* guinea pigs

These results suggest that the antibodies act as a carrier for the insulin

B Boeryd EXPERIMENTAL STUDIES ON THE EFFECT OF HEPARIN AND FIBROLYSIN INHIBITORS ON TUMOUR METASTASIS

B Stenkvist POTENTIATING EFFECT OF 20 METHYLCHOLANTRENE ON THE DEVELOPMENT OF ROUS SARCOMA

J Ponten IN VITRO NEOPLASTIC TRANSFORMATION OF HUMAN CELLS BY SIMIAN VIRUS 40 (SV 40)

I. Hägerstrand & F. Linell: SARCOIDOSIS IN AN AUTOPSY SERIES

(Published in Acta Med. Scand.)

Th. Berge: THE ARIAS-STELLA REACTION

It is not known how soon this reaction may occur. A case is reported where typical changes were found within 14 days after conception.

S. Szögi & Th. Berge: HEPATO-ADRENAL NECROSIS IN THE NEWBORN

A. Moberg & H. Arvidsson: ON THE EXISTENCE OF EXTRACARDIAL CORONARY ANASTOMOSIS OF IMPORTANCE FOR THE MYOCARDIAL BLOOD SUPPLY

Many postmortems reveal severe arteriosclerosis with almost total occlusion of the coronary arteries without major pathological changes in the myocardium. Can this discrepancy be due to extracardial anastomoses to the myocardium?

In coronary angiography one of us (H.A.) noticed wide vessels branching off from the internal mammary and/or bronchial arteries. These vessels seemed to *supply the heart and in a few cases contrast from the bronchial arteries seemed to* reach the coronary arteries.

In 12 cases postmortal angiography has been performed in the internal mammary arteries. In all cases pericardial vessels were filled but in only two cases minor vessels in the left atrium. No vessels in the ventricular walls were observed.

In 22 cases angiography was performed in the bronchial arteries. In 21 cases vessels in the right or left atrium were filled with contrast and in 9 of these cases vessels in the ventricular walls were also filled.

B. Robertson: THE PULMONARY ARTERIAL PATTERN IN TRANSPOSITION OF THE GREAT ARTERIES

Microangiographic and histologic studies of the pulmonary arterial pattern in 5 neonatal autopsy cases of complete transposition of the great vessels displayed wide and tortuous arteries with the localization and structure of bronchial arteries. These vessels, however, were found to be branches from the pulmonary artery, originating near the hilum. Like normal bronchial arteries they gave off branches to lymph nodes and to the bronchial mucosa, but they were also found to ramify in the pulmonary parenchyma. Many alveolar groups displayed a double vascularization, on one hand by fairly normal branches from the pulmonary artery, on the other by the above mentioned abnormal bronchial vessels.

In all cases the injection of contrast medium had been made into the transposed pulmonary artery, and therefore it cannot be settled whether there are also true bronchial arteries originating from the aorta, or whether the abnormal bronchial vessels represent transposed bronchial arteries.

O. Hassler: MICRORADIOGRAPHY AND MICROANGIOGRAPHY OF THE BRAIN. TECHNICAL POINTS

O. Hassler: LARGE MEDIA DEFECTS IN HUMAN ARTERIES

L Angervall P Bjorntorp I Nilsson B Stener & I Wickbom ANGIOGRAPHIC, MICROANGIOGRAPHIC, HISTOLOGIC AND CHEMICAL STUDIES ON HIBERNOMAS AND ORDINARY LIPOMAS

Two hibernomas (a benign tumour characterized by multivacuolated fat cells) and a number of ordinary lipomas (univacuolated fat cells) have been studied preoperatively with angiography and postoperatively with microangiographic, histologic and chemical methods For microangiography a technique was used which enables examination of the capillaries

The two hibernomas were more vascularized than ordinary lipomas In one of the cases preoperative angiography showed in addition to high vascularity a pronounced arterio venous shunting which is considered a contributary angiographic sign of malignancy The high vascularity of hibernomas is in keeping with the fact that brown fat—the assumed matrix of the tumour—contains more vessels than ordinary fat

In one case in which an ordinary intramuscular lipoma was studied microangiographically the vascular pattern suggested that bundles of muscle fibers passed through the tumour this was confirmed histologically

The hibernomas as well as brown fat from mice, contained more cholesterol and phospholipids in proportion to triglycerides than did ordinary lipomas Also with respect to the phospholipid composition the hibernomas differed from ordinary lipomas in the same way as brown fat

G Wihman CYTOLOGIC EXAMINATION OF SPUTUM—TECHNICAL POINTS

J Zajicek CYTOLOGIC DIAGNOSIS OF SALIVARY GLAND TUMOURS

K Mansson & F Linell PREVALENCE AND INCIDENCE OF CARCINOIDS

The prevalence of carcinoids was studied in an autopsy series of 4898 cases from the city of Malmo for the years 1959 1962 The cases came from the only hospital of the city with an autopsy frequency of about 93 per cent and they represent about 60 per cent of all deaths in the city (about 230000 inhabitants) during the period The prevalence of carcinoids in the gastrointestinal tract was 1 per cent and of bronchial carcinoids 0 1 per cent Most of the former were situated in the small intestine and 25 per cent had metastasized Only one case of 48 with carcinoids in the gastrointestinal tract which showed the carcinoid syndrome was clinically diagnosed All the other cases were incidental autopsy findings

The incidence of carcinoids in the gastrointestinal tract was studied in the operation material from the hospital during the years 1957-1962 It was estimated to be < 1 case per year and 100000 inhabitants The carcinoid syndrome was extremely rare and can be expected to occur in Malmo once in 10 years

In the operation material which were patients younger than 40 years most of the cases had appendix carcinoids In the autopsy material however which consisted mostly of very old patients no appendix carcinoids were found These findings indicate a very slow growth of carcinoids and that the carcinoids of appendix are discovered because of wide indications for operation in the region of appendix while the carcinoids of the small intestine are overlooked

J Mellgren G Moberger & J G Norden CLASSIFICATION OF PREMALIGNANT CHANGES OF PORTIO AND CERVIX UTERI

The Society recommended the following scheme suggested by *Norden*

PAD	*Clinical significance*	*Treatment*
Carcinoma in situ: undifferentiated / intermediate / spinocellular	Carcinoma in situ	Conisation (hysterectomy in older patients)
Strong atypia	Strong suspicion of carcinoma in situ	
Moderate atypia	Suspicion of *carcinoma in situ*	Control + treatment of inflammation if present
Slight atypia	No suspicion of carcinoma in situ	
Basal cell hyperplasia Leukoplakia Metaplasia	Benignant	

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HEATED FATS AND ALLIED COMPOUNDS AS CARCINOGENS

Studied by the Newt Test

By

E. Arffmann

Received 4 x 63

Extensive studies through decades have failed to clarify whether heated and oxidated fats have carcinogenic properties This appeared from a review of accumulated experimental results (*Arffmann* 1960) pointing to the incapability of the conventional methods of bioassay to yield conclusive evidence It seems therefore highly desirable to develop new methods for the demonstration of carcinogenicity which at the same time may be expected to reveal unknown effects of carcinogens A series of experiments on the newt test (*Arffmann* & *Christensen* 1961, *Arffmann* 1962, 1963 and 1964) has shown a specificity sufficient for a screening method if the chemical compounds are given in oil, which makes the method convenient for the study of heated fats and allied compounds

Heating and Oxidation of Fats

The chemical changes induced by heating of lipids depend on the presence or absence of oxygen, the active components being primarily the unsaturated fatty acids (*Axt* 1959) Most important is the oxidative transformation corresponding to autoxidation which has found excellent description in works by *Paquot* (1958), *Axt* (1959) and *Glavind* (1963) The first step is the formation of hydroperoxides [illegible]

The author s thanks are due to Director cand polyt [illegible] and cand polyt [illegible] the chemistry of [illegible] author is indebted [illegible] his experienced [illegible] and for inspiring

cular-weight unsaturated carbonyl compounds Also epoxides are formed late in the process

If oxygen is absent during heating oxygen-free polymers, often of a cyclic structure, are formed Decomposition products are paraffins aldehydes, acids and ketones

The most simple analytical methods to follow the oxidation are determinations of the peroxide value and of the carbonyl (aldehyde) value The peroxide value expresses the actual quantity of hydroperoxide groups and is therefore not a measure of the total oxidation (*Deutsch et al* 1941) *The carbonyl value measures the secondary* oxidation products, which are determined as aldehydes (*Holm et al* 1957)

The present knowledge of the chemical changes in heating and oxidation of fats is, however, only elementary In summary, complicated processes of oxidation, polymerization and decomposition lead to a vast mixture of reaction products, including polymers, peroxides, aldehydes ketones, epoxides, lactones and free fatty acids (*Wurziger & Osterlag* 1960)

Heating of cholesterol probably leads to oxidation, and the formation of ketonic steroids is suggested by *Haven & Bloor* (1956) The products *may possess hydroperoxide- or epoxide groups* (*Fieser* 1954)

MATERIAL AND METHODS

Substances Soyabean oil was chosen as test object for the study of heated fats This oil is used for the preparation of edible emulsifiers especially for the manufacture of margarine and baking compounds and besides its content of the more unsaturated fatty acids is rather high

Simple heating was performed on an infrared radiation bath (BB Infrabath Bie & Berntsen Copenhagen) furnished with a relay and a contact thermometer The fresh soyabean oil (supplied by Dansk Sojakagefabrik) was contained in a Pyrex beaker or retort and the contact thermometer put into the oil A temperature of 350° C was reached after 45 to 60 minutes and maintained for exactly ½ or 1 hour During this time the temperature was not below 350° C and not above 355° C After the heating period the oil was immediately removed from the warm plate Air had free admittance but was not bubbled through The end product was a dark brownish and distinctly viscous material

Some of the soyabean oil was heated in nitrogen atmosphere The fresh oil was bubbled through with nitrogen for 6 hrs 20 min before being poured into a retort which was sealed from the surrounding air The warm up time was 105 minutes and a strong current of nitrogen through the retort was maintained until 300° 320° C was reached after about 70 minutes The sealed system was heated at 350° 355° C for exactly 1 hour The nitrogen current was reestablished during the first hour of cooling and then the retort was closed The end product was of a lighter colour and less viscous than that after heating in the presence of air

Chemical data of the polymerized soyabean oils are given in Table 1

The viscosity of the heated oils made it necessary to dilute the products with 3 parts of fresh soyabean oil (v/v) before injection

Commercially polymerized soyabean oil was kindly supplied by Grindstedvaerket Viby J Denmark *It is used in an edible emulsifier and produced by heating at 300° C in vacuum* (about 20 mm Hg) for about 48 hours until the desired viscosity has

Milan)

mono

r weight

meric acids

of the monomeric glycerid fraction was about 920 while total oil molecular weight was 2470–3120 Because of the viscosity the product was administered as a 25 per cent—in one experiment 50 per cent—solution in fresh soyabean oil (w/w)

TABLE 1

Chemical Analyses of Heat Polymerized Soyabean Oil

Substance	Peroxide value m e kg	Carbonyl value mg%	Iodine value	% Urea filtrate	% Non distillable methyl esters
				Acids	
Soyabean oil ..	1 4	6 2	131	6	5
Soyabean oil heated in N atm at 350° C for 1 hr	4 7	217	75	58	53
Soyabean oil heated in air at 350° C for 1 hr	5 1	206	73	66	56
Soyabean oil heated in air at 350° C for ¾ hr					51

The peroxidized soyabean oils were prepared by the Laboratory at Grindsted Vaerket *Moderate heating* vigorous stirring *of the oil* and a constant current of air through the retort were maintained until the peroxide content had risen to the highest obtainable values Before that specimens had been taken out at lower levels *with proper intervals In experiments Nos 11 and 12 (Table 3)* the P V 1 4 5 oil was oxidated at 37° C for 131 hours while the P V 858 specimen was made at 100° C for 21 hours The same temperature was applied to the P V 293 oil through 28 hours, but after the oxidation this specimen was mixed with equal part of fr...

oils were quickly heated at ... minutes at 10 mm Hg in order to destroy the peroxides These samples are designated B in the tables

The peroxidized soyabean oils were—with a few exceptions—injected without any dilution

Ethyl l ...

and di

¹ P V = peroxide value determined a m Wheeler (DGF Einheitsmethoden C VI 6a (57))

Experiments wit

…ent No	35-36			40								
…nce	Soya bean oil	Soya bean oil heated in air at 350° C for 1 hr 25% in soya bean oil	Dibenz [a,h]anthracene 0.1% in soya bean oil	Soyabean oil*		Soyabean oil heated in N atm at 350° C for 1 hr 25% in soyabean oil		Soyabean oil heated in air at 350° C for 1 hr 25% in soyabean oil		Soyabean oil		Soya… hea… N atm… fo… 2… soya…
…lication	Tail		Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail
…animals …status)	4	8	8	4	4	4	4	4	4	4	4	4
	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
…reac…on on												
…d ty		–	–									
	–	–			–						–	–
	–	–	– +	–	–		–	–		–	–	
	–	– –	+	–								
		– –	+	–	–		–		–			
…died …eriment	0	1 (included)	3 (2 included)	1	0	3 (1 included)	1	2	0	0	0	2 (in clu le…)

…ted because of epidermolysis

occurring product in estrogen metabolism is closely related to oestradiol which was chosen for parallel control and kindly supplied by the Leo Pharmaceutical Products Both substances were found difficult to solve in peanut oil requiring vigorous stirring and heating on waterbath (<100° C) for 5 to 11 minutes to obtain 0 5 per cent solutions In most instances slight secondary precipitation after cooling was eliminated before injection by heating over open fire for a few seconds

Cholesterol was obtained from The British Drug Houses Ltd (Laboratory Reagent Melting point 145° to 149° C Specific rotation $(\alpha)\ {}^{20}_{D}$ (in chloroform) —38° 5 to —40° 5 Moisture Not more than 0 5 per cent Sulphated ash Not more than 0 1 per cent) Cholesterol was heated on the BB Infrabath the compound being placed in a Pyrex glass which together with the contact thermometer was put into a beaker with silicon oil One sample was heated at 350° C another at 200° C both for exactly 1 hour The warm up time was 30 and 14 minutes respectively After the 350° C heating cholesterol appeared darkbrown to black and after cooling was paste like The lower temperature changed the white colour to light yellow brown Both specimens were like untreated cholesterol easily solved in oils by the use of stirring and heating on waterbath (<100° C) for 1 1½ minutes

...ymeri ed Soyabeon Oil

		49				50			51			52	
	...abean oil heated in air at 350 C for 1 hr 2..% in ...abean oil	Soya bean oil	Soya bean oil heated in N atm at 350 C for 1 hr 25% in soya bean oil	Soya bean oil heated in air at 350 C for 1 hr 25% in soya bean oil	Benzo(a)pyrene 0.5% in soya bean oil	Soya bean oil	Commercially polymerized soya bean oil 25% in soya bean oil	Benzo(a)pyrene 0.1% in soya bean oil	Soya bean oil	Commercially polymerized soya bean oil 25% in soya bean oil	Benzo(a)pyrene 0.1% in soya bean oil	Soya bean oil	Commercially polymerized soya bean oil 50% in soya bean oil
1	Back	Tail				Tail			Tail			Tail	
	4	8	8	8	8	8	8	8	8	8	8	8	8
♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
	–	– – – –	–		+ – – +	– – – – –	• – –	– – (+) –	– – – –	– – –	+ (+) + (+) (+) – –	– – – – – –	– – – – – – –
1	1	3	2 (included)	3 (1 included)	2 (1 included)	0	2	1 (included)	1	1	1 (included)	0	0

Techn cal ...

RESULTS

The results are shown in Tables 2 10 Negative and positive controls are included The various heat polymerized soyabean oils gave a negative outcome in all experiments (Table 2)

Strongly peroxidized soyabean oil was submitted to two tests (Table 3) The results are slightly varying, probably because of a less reliable technique in these early experiments, but indicate clearly a weak or moderate positive reaction to the two oils with a peroxide value of 858 and 203 m e/kg respectively In contrast, the P V 4 5 oil gave a result not different from that seen after fresh oil, while heavy dilution (2 per

TABLE 3

Experiments with Strongly Peroxidized Soyabean Oil

Experiment No.	11															
Substance	Peanut oil		Perox soya bean oil (P.V.* 45)		Perox soya bean oil (P.V. 293)		Perox soya bean oil (P.V. 858)		Perox soya bean oil (P.V. 45) 2% in peanut oil		Perox soya bean oil (P.V. 293) 2% in peanut oil		Perox soya bean oil (P.V. 858) 2% in peanut oil		3-Methylcholanthrene 0.1% in peanut oil	
Site of application	Tail															
Number of animals (Triton cristatus)	7		7		7		7		7		7		7		7	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal reaction on																
3 day													–			
6	–					(+)		–				†			(+)	
9						+			–					(+)	+	
12										†					–	
15						+			–					–		+
18				–	+		†		–					–		+
21													–			
Animals died during experiment	0		0		0		0		0		0		0		0	

Experiment No.	12																	
Substance	Soya bean oil		Perox soya bean oil (P.V. 4)		Perox soya bean oil (P.V. 293)		Perox soya bean oil (P.V. 858)		Perox soya bean oil (P.V. 45) 2% in soya bean oil		Perox soya bean oil (P.V. 293) 2% in soya bean oil		Perox soya bean oil (P.V. 858) 2% in soya bean oil		3-Methylcholanthrene 0.1% in soya bean oil			
Site of application	Tail																	
Number of animals (Triton cristatus)	7		7		7		7		7		7		7		7			
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal reaction on																		
3 day		+	(+)			–		±		–		–		+		–		
6			–		–			+		–	–			†		–		
9				†		–	+			–		–	–		+			
12			–				+			–	–			–	(+)			
15	–			–			–		–			–	(+)		+			
18				–		+	–			–	–			§	+			
21	–			–	–			+	–		–		–		–			
Animals died during experiment	0		0		0		0		0		0		0		0			

* P.V. = peroxide value (m.e./kg)
† No signs of the injected oil
§ Sections technically bad

TABLE 4

Experiments with Peroxidized Soyabean Oil before (A) and after (B) Heat Reduction

Experiment No			45	46	47	49	50	51	52	54	69	70
Substance	PV*	CV§	Positive reactions/Total number of animals									
Oil 0	1 9	12 5	1 5	0 5	0 6							
Oil 0	2 2	3 4				0 8	0 8	0 7	0 8			
Oil 0	1 4	10 2								1 6		
Oil 0	3 0	2 5									1 8	0 8
Oil 1A	33 0	9 5	0 7	0 6								
Oil 2A	55 0					0 8				0 6		
Oil 3A	78 0	9 1	0 6	0 6								
Oil 4A	104 0					0 8				0 6		
Oil 5A	161 0	13 1	2,7	1 6								
Oil 6A	193 0					0 8		1 8		0 6		
Oil 7A	236 0	22 0	0 2	0 5	0 4							
Oil 8A	362 0					0 8	0 8	0 8	0 8	0 6		
Oil 9A	407 0	57 2									2 8	3 16
Oil 1B	1.5	47 0	0 3	0 5								
Oil 2B	0 6	67 0				0 8				0 5		
Oil 3B	1 8	90 0	0 6	2 5								
Oil 4B	0 7	137 0				0 8				0 5		
Oil 5B	2 6	169 0	0 6	0 6								
Oil 6B	1 3	310 0				0 8		0 7		0 6		
Oil 7B	4 1	165 0	0 3	0 6	0 4							
Oil 8B	3 8	640 0				1/8	0 7	0 8	0 8	0 6		
Oil 9B	3 1	338 0									2/7	3/16
Dibenz(a,h)anthracene 0 1%			0 2									
Chrysene 0 5%				2 5					3 8			
Benz(a)pyrene 0 1%					3 6		1 8	4 8			5/7	
0 5%												8 8
Benz(a)anthracene 0 1%						0 6						
0 5%										2 5		

* PV = peroxide value (me/kg)
§ CV = carbonyl value (mg%)

cent) of the PV *808 and 293* oils weakened the positive response distinctly

The following extensive experiments on the reactivity of the newt epidermis *to peroxidized soyabean oil are* summarized in Table 4 *The* oil numbering is arbitrary *and in* series A refers to the increasing peroxide value of the *samples while* series B represents the same specimens after heat *destruction* of the peroxides O oils are untreated fresh soyabean oils *Positive* reactions cover + and (+) responses while doubtful *reactions* (±) *are not* included Total number of *animals* represents *effective total Eleven* positive reactions to soyabean oils with a peroxide value *of 161 or more* were seen among 108 newts With one exception all of these *responses fall* in experiments 4a–47 being made in the autumn *and* in experiments 69 70 using hibernating *animals* from the *preceding year's batch* which in both cases *means* that

perlment No	47										
Substance	Soya bean oil 0* (P V 1 9 C V 12 5)†	Soya bean oil 7B (P V 4 1 C V 16.5)	(P V 206 C V 22)	in soya bean oil 0	in soya bean oil 7B	in soya bean oil 7A	in soya bean oil 0	Soya bean oil 0 (P V 3 0 C V 2 5)	Soya bean oil 9B (P V 3 1 C V 33)	Soya bean oil 9A (P V 409 C V 57 2)	B a r 0 0 s l o
of application	Tail										T
ber of animals llon cristatus)§	6	6	6	6	6	6	6	8	8	8	
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	C
Epidermal reaction on											
3 4 day		−						(+)			
6											
9										−	+
12	−		−	− −	(+) −	(+) −	+ (+)	−		− +	−
14 15	−	−	+ (+)	− (+)	+	+	+	−	(+)	− −	
18	−			+ −	+ −		−		(+)	− +	+
nimals died ng experiment	1 (in cluded)	3 (1 in cluded)	2	0	0	0	0	2 (in cluded)	2 (1 in cluded)	0	(cl

numbering of the oils is the same as in Table 4
' = peroxide value (m e/kg) C V = Carbonyl value (mg$^{C}_{c}$)
riton marmoratus in exper no 47

the animals have been in a rather sensitive state (cfr *Arffmann* 196
Experiments 49 54, holding only one positive response, were made
the spring on newly received and therefore more resistant anim
This is reflected by the positive controls in experiments 49 50 In th
B series the result of experiments 69 70 equals that in the A seri
Besides, the positive reactions are few, but it is difficult to explain wh
2 out of 5 animals in experiment No 46 react to an oil with carbon
value 90 considering the many negative responses at higher carbon
values

A possible cocarcinogenic effect of oxidated soyabean oil was e
amined in three experiments shown in Table 5 The first experime
(No 47) was indicative of such an effect, but could not be confirme
Unfortunately an unexpected high reactivity to benzpyrene was se
at a concentration of 0 025 per cent, and signs of promotion could n
be demonstrated, when oxidated soyabean oil was used as vehicle in
stead of the fresh oil

The first experiment with ethyl linoleate hydroperoxide is n

t of Oxidated Soyabean Oil

		70												
o- y e %, a n A	Benzo-(a)pyrene 0.1%, in soyabean oil 0	Soyabean oil 0 (P.V. 3.0 C.V. 2.5)		Soyabean oil 9B (P.V. 31 C.V. 3.6)		Soyabean oil 9A (P.V. 409 C.V. 5.2)		Benzo-a-pyrene 0.025%, in soyabean oil 0		Benzo-a-pyrene 0.025%, in soyabean oil 9B		Benzo-a-pyrene 0.025%, in soyabean oil 9A		Benzo-pyre 0.5% in soyabe oil (
		Tail	Pr T ††	Tail	Pr T	Tail	Pr T	Tail	Pr T	Tail	Pr T	Tail	Pr T	Tail
	8	5	3	8	8	8	8	5	3	5	3	5	3	5
♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
−	+	−	−	−	− +	∓	− −		−	+	−		−	−
	••		−	∓	− −	−	− −	+	−	+	+	−	+	+
+ +	+ +	− −		−	− −	∓	− −	+ +		+ −		− +		+ −
+ +	∓	−		+ +	− −	∓	− −	±	−	+	−	+	−	−
	0	0	0	0	0	1 (included)	1 (included)	1		0	0	1	0	0

Unsuited because of heavy skin infection
Part of tail proximal to secondary tail

tabulated Since the preparation of the compound several months earlier the peroxide value had fallen from 3000 to 2000 m e/kg The oil was injected undiluted into 11 Triton marmoratus and 1 Triton cristatus From the 4th day severe toxic lesions with extensive necrosis and deep ulceration were seen in the tails, so that the experiment ha[illegible]

[illegible] extent degree with extensive and strong hyperplasia Less striking was the slight or moderate infiltrative downgrowth at the edges or in the bottom of the ulceration The severe inflammatory lesions made it impossible to estimate the epithelial reaction exactly in terms of degree, but the changes strongly indicated further experimentation

Tables 6 and 7 show the results with ethyl linoleate hydroperoxide of peroxide value 750 and 1500 m e/kg and in various concentrations, compared to the effect of reduced ethyl linoleate hydroperoxide and pure ethyl linoleate The degree of toxic symptoms was practically equal

Experiments with Ethyl Li

Experiment No	54				57				
Substance	Soya bean oil	Ethyl lino-*leate* hydro per-oxide 750 m e kg	Ethyl lino *leate* hydro per-oxide 1500 m e kg	Benz [a in thra cene 0 5% in soya bean oil	Soya *bean* oil	Ethyl *lino-*leate	Dibenz [a h an thra cene 0 025% in soya bean oil	Peanut oil	Ethyl lino-l ate hy lro-per oxi le 750 m e kg 50% in peanut oil
Site of application	Tail				Tail				
Number of animals (*Triton cristatus*)	6	6	6	6	8	8	8	8	8
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
Epidermal reaction on									
5 7 day		♂ •	♂ •					♂ —	♂ —
8 9	♂ +	♀ •	♀ •	♂ —	♂ —, ♀ —	♂ (+), ♀ —	♀ +		♀ —
12 13	♂ —, ♀ —	♂ +, ♀ +	♂ + +, ♀ • +	♂ +, ♀ —	♂ —	♀ —	♂ +, ♀ +	♂ —, ♀ —	♂ •, ♀ +
14 15	♀ —	♂ (+), ♀ •		♂ —, ♀ —	♀ —		♀ +	♀ —	♂ —, ♀ (—)
18 19				♀ †	♂ —, ♀ —	♂ —	♂ +		
Animals died during experiment	0	1 (included)	0	0	0	1 (included)	0	0	0

* Severe necrosis † Sections technically bad

for the two peroxides (Fig 1) and the reduced form, but decreased with concentration, being absent at 10 per cent Undiluted ethyl linoleate had negligible toxicity A few cases (in experiments 54 and 55) could not be classified because of severe necrosis Ethyl linoleate hydroperoxide P V 1500 induced a strong, positive reaction in the newt when undiluted and as a 50 per cent solution in peanut oil (Fig 2) The response decreased with the concentration and was totally negative at 1 per cent The reaction to the reduced compound was a little less but also positive Ethyl linoleate hydroperoxide P V 750 induced a clearly weaker positive response than the P V 1500 compound, and pure ethyl linoleate can be estimated as negative

The toxic lesions being similar to those observed after the injection of tributyrin made it interesting to compare the latter compound with ethyl linoleate hydroperoxide *in a few experiments Toxicity showed* to be of the same order In one experiment (No 55) a 50 per cent solu-

droperoxi le (High Concentrations)

...ribu yrin 50% in eanut oil	As_2O_3 in peanut oil (0.5% suspension)	Peanut oil	Ethyl linoleate 50% in peanut oil	Reduced ethyl linoleate hydroperoxide 1500→0 me/kg 50% in peanut oil	Ethyl linoleate hydroperoxide 750 me/kg 50% in peanut oil	Ethyl linoleate hydroperoxide 1500 me/kg 50% in peanut oil	Dibenz a h anthracene 0.012% in peanut oil	Peanut oil	Ethyl linoleate 2% in peanut oil	Reduced ethyl linoleate hydroperoxide 1500→0 me/kg 2% in peanut oil	Ethyl linoleate hydroperoxide 1500 me/kg 2% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil
				58						61		
				Tail				Tail				Back
8	8	8	8	8	8	8	8	4	8	8	8	8
♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
•	−					∓						
(+)	(+)			(+)	− −	−	− +	−	− −	− ±	+ (+)	+ +
		−		+ −	− −	+ +	+ −	−	− −	+ −	− −	− +
−	− ±	−	− −	+	− −	+ +	+ −	−	− −	− −	+	+ +
		−	−	(+) −	+ −	+	+ +	−	− −	− −		+ (+)
0	1 (included)	1	1	0	0	1	0	0	0	0	1	0

tion (s/c) of tributyrin in peanut oil had the same carcinogen-like effect as ethyl linoleate hydroperoxide P.V. 750, and even at 10 and 1 per cent single positive reactions were seen. This result seems to indicate that tributyrin is not a totally indifferent compound (cfr. *Arffmann* 1962). Peroxide value of the tributyrin was 0 and carbonyl value 1.4.

A completely negative result followed the injection of epoxidized soyabean oil in three experiments (Table 8). The same applies to 16 α, 17 α epoxyoestratrien 3 ol which was submitted to three separate tests comprising 24 animals with intact tails. Also estradiol appeared next negative, while 0.5 per cent benzo(a)pyrene in the positive control experiments provoked epithelial proliferation in 20 out of 24 animals.

In contrast, the testing of a low-molecular diepoxide, diepoxybutane, showed positive reaction to all isomers (Table 9). A preliminary experiment (No. 65) with dl diepoxybutane revealed a moderate irritative

TA[BLE]

Experiments with Ethyl Linol[eate]

Experiment No	59							
Substance	Peanut oil	Ethyl lino leate 10% in peanut oil	Ethyl lino leate hydro per oxide 1500 mg/kg 10% in peanut oil	Reduced ethyl lino-leate hydro per-oxide 1500→70 mg/kg 10% in peanut oil	Dibenz [a h]an thra cene 0.0025% in peanut oil	Peanut oil	Tributyrin 1% in peanut oil	10[%] in pea[nut] oi[l]
Site of application	Tail							
Number of animals (Triton cristatus)	8	8	8	8	8	8 (Tr crist & vulg)	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂
Epidermal reaction on								
5 7 day								
8 9		− −	− +	−	−			−
12 13		− −	− +	− +	+ +		−	−
14 15		− −	− −	−	+ +	− −	+ −	−
18 19			− −	−	−		−	−
Animals died during experiment	0	0	0	0	0	0	0	0

effect at a concentration of 10 per cent, but at 1 and 0 5 per cent such lesions were not seen In two out of three experiments the positive reaction to the meso-compound was slight, but else there was no significant difference between the various isomers (Fig 3)

In seven different experiments 0 1 or 0 5 per cent solutions of cholesterol induced no epithelial reaction in newts After heating of the cholesterol at 200° C for 1 hour, one single positive response occurred among 16 animals, injected with 0 5 per cent solutions Preheating at 350° C, however, formed a product which in the same concentration provoked epithelial reaction in about half the animals (Fig 4) Experiment No 66 confirmed this finding, being made 7 months later A 0 1 per cent solution of the heated cholesterol had no effect (Table 10)

7
Hydroperoxide (Low Concentrations)

					60				
Ethyl linoleate hydroperoxide 50 m e kg 1% in peanut oil	Ethyl linoleate hydroperoxide 50 m e kg 10% in peanut oil	Ethyl linoleate hydroperoxide 1500 m e kg 1% in peanut oil	Ethyl linoleate hydroperoxide 1500 m e kg 10% in peanut oil	Benzo-[a py rene 0 5% in peanut oil	Peanut oil	Ethyl lino-leate 1% in peanut oil	Reduc ethyl lino-leate hydro-per-oxide 1500→70 m e kg 1% in peanut oil	Ethyl lino-leate hydro-per-oxide 1500 m e kg 1% in peanut oil	Benzo-[a py rene 1% in peanut oil
Tail					Tail				
8	8	8	8	8 (Tr crist & vulg)	4	8	8	8	8
♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
	– – – – –	– – – – – –	– – – – – – – –	– + – + – + + +	– – – –	– – – – – – – –	– ± – – +	– – – – – – – –	+ + – + + + + +
1	1	1 (in clude l)	0	0	0	0	0	1 (in-clu led)	0

DISCUSSION

The present results with peroxides suggest a weak carcinogenicity of such compounds Most impressing was the reaction to ethyl linoleate hydroperoxide at high peroxide value and in high concent ations, while ... This differ- ... a causality ... quantitatively different response ... eriments Nos 11 and 12, and by the oxides (P V 1500 and 750) The results may, however, have been influenced by the fact that the linoleate peroxide ... cular, more ... the peroxy ... less mobile ... toxic reaction to ethyl linoleate hydroperoxide is another expression of this circumstance and may have added to the

epithelial response The carcinogenicity of peroxides is considered weak, because positive reaction demands a very high peroxide value of the oil or the use of sensitive animals

The positive results obtained with reduced peroxides indicate that the activity is connected with other oxidation products as well, even if *peroxy groups seem to be the most active* The carbonyl value is a summarized expression of the secondary oxidation products in the thermo-reduced soyabean oils (B series), while reduction of ethyl linoleate hydroperoxide probably leads to the formation of alcohol groups (*Glavind* 1963) The exact nature and quantities of these various reaction products is not known

That the observed effect is connected with oxidation of the oils is supported by the other results of the present experiments Heating of cholesterol is supposed to yield oxidative transformation products (*Fieser* 1954, *Haven* & *Bloor* 1956), and cholesterol heated at 350° C provoked a positive response in newts Diepoxybutane likewise showed a distinct activity, while the negative result with epoxidized soyabean oil may be explained by the high molecular weight and low mobility of the active groups The results with 16 α, 17 α epoxyoestratrien-3 ol may express inactivity of monoepoxides

Commercially polymerized soyabean oil is heated in vacuum, and the soyabean oil heated at 350° C in the presence of air showed only a mild degree of oxidation, the carbonyl value not exceeding that seen after heating in nitrogen atmosphere Also *Wurziger* & *Osterlag* (1960) found a moderate rise in carbonyl value on heating of oils in a current of nitrogen Table 4 shows that with a few exceptions oils in series B with a carbonyl value below 300 gave no positive reactions Furthermore, the high-molecular-weight of polymerized oils will restrain the effectivity of any oxidized groups So the negative effect of heat-polymerized soya bean oil is in accordance with the hypothesis that oxidation is the major factor responsible for the carcinogen-like activity of some heated lipids in newts

---

Figs 1 4

Fig 1 Large ulcerative defect with necrotic tissue remnants on 12th day after injection of ethyl linoleate hydroperoxide (P V 1500) 50 per cent in peanut oil Strong epithelial hyperplasia is seen at the edge of the ulceration and on the other side of the tail (10 ×)

Fig 2 Epithelial hyperplasia and infiltrative downgrowth on 19th day after injection of ethyl linoleate hydroperoxide (P V 1500) 50 per cent in peanut oil (40 ×)

Fig 3 Strong epithelial hyperplasia and infiltrative downgrowth on 15th day after injection of 1 diepoxybutane 1 per cent in peanut oil (40 ×)

Fig 4 Epithelial hyperplasia and downgrowth on 18th day after injection of heated cholesterol (350° C for 1 hour) 0 5 per cent in peanut oil (40 ×)



Fig 1 4

TABLE 8
Experiments with Epoxidized Soyabean Oil

Experiment No	39						10				41						
Substance	Soyabean oil		Epoxy soyabean oil 25% *in* soyabean oil		Benz a anthracene 0.1% *in* soyabean oil		Soyabean *oil*		Epoxy soyabean oil 25% in soyabean oil		Soyabean *oil*		Epoxy soyabean oil 25% in soyabean oil				
Site of application	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back			
Number of animals (Triton cristatus)	4	4	4	4	4	4	4	4	4	4	4	4	4	4			
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																	
5 day						+											
9	–				–			–	–	–	–	–		–			
11 12	+			–			–	–	–	– –	–	–		–			
15				–	+	–	–	–		–	–	–	–	–			
18	±	–		–	+	–	–	–	–		–	–		–			
Animals died during experiment	0	0	0	0	0	1 (included)	1	0	0	2 (included)	0	0	3	0			

TABLE 9

Exper e ts th D e) apl t ne

Experim t N	r		0					17					68					
S l t n e	10 % in o l	p x y e l n o l	f a t oil	epoxy ane 0 % pen l	ep xy l u n e 0.5 % p a o l	epoxy f ta e 0.5 % f a u	e a r e 0 % pen ul	a t o l	epoxy ta e 1 % a u	epoxy e 1 % p a o l	p xy a e 1 % ut	e ro a y r 1 % n t	ea t o l	xy 1 % pen	ep x 1 % n a o l	e xy e 1 % re	e x ta e 1 % n	a y r e 1 % rea t o l
Sl e of appl ca i n	La f al proximal o s con f ry tail		[illegible]	Lat f ta l prox o s c lary ll				La t of t ll prox s co lary tail					Lat f t ll r xi l o r y t ll					
N t r of a (l r r s)	6	6	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
E i ler mal re c on																		–
6 day												+ +	– +	+ –		– +		+
9	(+)		–	–														
12	+ ( )		– –	( ) – (+			+		( )( )	+ +	+	–	– –	–	– –	( )		+ +
1	( ) –	– (			+	(			( )	±	( )( )	+ –		(+)	+	+		+
18 19	–	+	–	( )					+	(+)	+	+		( )( )	–	+	–	+
N i als lie l r g experi	0		0	(	0	0	0	0	0	0	0	0	0	0	0	·	3 (1 1 el 1 l)	1 (1 el le l)

ent No	38		11			5[illegible]				8		
ance	Pea nut oil	Cholesterol 0.5% in peanut oil	Soya bean oil	Cholesterol 0.5% in soya bean oil	Dibenz a h anthracene 0.1% in soya bean oil	Soya bean oil	Cholesterol 0.1% in soya bean oil	Cholesterol heated at 350 C for 1 hr 0.1% in soya bean oil	Dibenz a h anthracene 0.025% in soya bean oil	Pea nut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 350 for 1 0.5% in peanut oil
lication	Tail		Part of tail proximal to secondary tail			Tail				Tail		
f animals (istatus)	6	6	6	6	6	8	8	8	8	8	8	8
x	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
l reac ion on												
9 day							– –		+ +	–	–	–
2		– –	–	–	+	– –	– –	–	+ +	–	– –	+
5	–	–	–	– –	+	–	–	–	+ +	– –	–	+
8	–	– –		–	+ +	– –	–	–	+ –	–	– –	[illegible]
s died eriment	0	0	0	0	0	0	0	0	0	1	0	0

mal signs of the injected oil

SUMMARY

Some heated and oxidated fats and a few allied compounds were tested in newts. Positive results of varying degree were obtained with peroxidized soyabean oil and ethyl linoleate hydroperoxide, but the carcinogen-like activity of the peroxides is considered rather weak when the response is compared with the magnitude of peroxide values and the sensitivity of the animals. Reduction of the peroxides through heating or by chemical means weakened the activity only slightly. Epoxidized soyabean oil and 16 α, 17 α-epoxyoestratrien-3-ol induced no epithelial reaction, while the testing of diepoxybutane proved positive for all isomers. Heating of cholesterol at 350° C for 1 hour gave a newt-positive product while untreated cholesterol and moderately heated cholesterol (200° C for 1 hour) were negative. Commercially polymerized soyabean oil and soyabean oil heated at 350° C for ½ or 1 hour had no effect on the skin of newts. It is concluded that oxidation may *be considered* the most important process in the transformation of lipids leading to newt-positive and therefore carcinogen-suspected products.

eated Cholesterol

	59			66			60				61			
t	Cholesterol 0.5% in peanut oil	Cholesterol heated at 300 C for 1 hr 0.5% in peanut oil	Dibenz a h anthracene 0.0025% in peanut oil	Peanut oil	Cholesterol heated at 300 C for 1 hr 0.5% in peanut oil	Benzo-a pyrene 0.5% in peanut oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 200 C for 1 hr 0.5% in peanut oil	Benzo-a pyrene 1% in peanut oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 200 C for 1 hr 0.5% in peanut oil	Be [a] re 0 pe
	Tail			Part of tail proximal to seconlary tail			Tail				Tail			B
	8	8	8	8	8	8	4	4	8	8	4	4	8	
	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂
		—	—	—	—	+ +	—	—	—	+ +	—	—	— —	+
		+ ±	+ +		+ •	+ +	—	—	— —	+ +	—	—	+ —	—
	—	+ (+)	+ +	—	(+)	+ —			— —	+ +		—	—	+
			—	— —	+ —	+ +	—		—	+ +		—	— —	+
	0	0	0	0	1	0	0	0	0	0	0	0	0	

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Experiments with Cho

...ent No	38		44			5[illegible]				[illegible]8		
...ance	Peanut oil	Cholesterol 0.5% in peanut oil	Soya bean oil	Cholesterol 0.5% in soya bean oil	Dibenz a h anthracene 0.1% in soya bean oil	Soya bean oil	Cholesterol 0.1% in soya bean oil	Cholesterol heated at 350° C for 1 hr 0.1% in soya bean oil	Dibenz a h anthracene 0.025% in soya bean oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 350° C for 1 hr 0.5% in peanut oil
...plication	Tail		Part of tail proximal to secondary tail			Tail				Tail		
...f animals (...ristatus)	6	6	6	6	6	8	8	8	8	8	8	8
...x	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
...l reaction on												
9 day						–	–	–	+ +	–	–	– –
12	–	– –	–	–	+	– –	–	–	+ +	–	– –	+ –
15	–	–	–	–	+	– –		–	+ +	– –	– –	– –
18		– –		– –	+ +	– –	– –	–	+		– –	+ +
...s died ...periment	0	0	0	0	0	0	0	0	0	1	0	0

...mal signs of the injected oil

SUMMARY

Some heated and oxidated fats and a few allied compounds were tested in newts. Positive results of varying degree were obtained with peroxidized soyabean oil and ethyl linoleate hydroperoxide, but the *carcinogen-like activity of the peroxides is considered rather weak* when the response is compared with the magnitude of peroxide values and the sensitivity of the animals. Reduction of the peroxides through heating or by chemical means weakened the activity only slightly. Epoxidized soyabean oil and 16 α, 17 α-epoxyoestratrien 3-ol induced no epithelial reaction, while the testing of diepoxybutane proved positive for all isomers. Heating of cholesterol at 350° C for 1 hour gave a newt-positive product, while untreated cholesterol and moderately heated cholesterol (200° C for 1 hour) were negative. Commercially polymerized soyabean oil and soyabean oil heated at 350° C for ½ or 1 hour had no effect on the skin of newts. It is concluded that oxidation may be considered the most important process in the transformation of lipids leading to newt-positive and therefore carcinogen-suspected products.

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GM CHARACTERS OF 7 S γ MYELOMA PROTEINS AND CORRESPONDING INDIVIDUAL NORMAL γ GLOBULINS[1]

By

ULF NILSSON*

Received 11 x 63

The presence of genetically determined characters Gm groups, in human γ globulin was first demonstrated by *Grubb & Laurell* (1956) Several additional gentic characters of γ globulin have been reported by other investigators (*Harboe & Lundevall* 1959, *Harboe* 1959a *Steinberg et al* 1960 *Brandtzaeg et al* 1961, *Ropartz & Lenoir* 1961, *Thomas & Kompf* 1961 *Steinberg et al* 1962) Grubb s data indicated that the Gm groups are properties of γ globulin of the 7 S variety (*Grubb* 1959), which was confirmed by *Martensson* (1961) *Fahey & Fowler* (1961) and *Harboe et al* (1962a), who examined γ globulin of the myeloma and macroglobulinaemia type (M components)

Mårtensson (1961) also obtained evidence for differences in Gm characters between M components and the corresponding unfractionated serum This observation led to the present study, in which sera were investigated as to the Gm type of their isolated M component and of the residual normal γ globulin A similar study has recently been carried out by *Harboe et al* (1962b) It was found that the Gm properties of the M components and the corresponding normal γ globulins differ widely The possibility will be discussed that the relative frequency of the Gm characters among M components reflects the molecular heterogeneity of normal γ globulin, which in turn might be an expression of heterogeneity existing among plasma cell clones

MATERIALS AND METHODS

Sera with M components demonstrable on paper electrophoresis were obtained from various hospitals in Southern Sweden and were stored at —18° C until processed The M components were classified with specific rabbit antisera to human β_{2A}, β_{2M} and 7 S γ-globulin Only those M components that were of the 7 S γ type were utilized in this study

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RESULTS

The isolation of both normal γ globulin and M-components from the same sera was only possible in 20 out of 35 cases. The remaining sera contained normal γ globulin in amounts too small to be isolated, and therefore in these cases the Gm type of the M components was determined on unseparated sera.

The isolated fractions of the former group of sera were subjected to paper, starch, and immunoelectrophoresis. Antisera specific to β_{2A} and γ globulin were used. With few exceptions, the M components were electrophoretically homogeneous and the normal γ-globulin preparations showed no obvious contamination with M components. γ globulins derived from fractions of faster electrophoretic mobility contained no demonstrable β_A-globulin. The presence of β_{2M}-globulin was excluded on the basis of the chromatographic conditions.

Four preparations of residual normal γ-globulin, obtained from myeloma sera, contained minor amounts of M components. Similarly, six of the isolated M-components showed minor electrophoretic heterogeneity due to the presence of small quantities of normal γ globulin.

Figs. 1, 2 and 3 show the agglutination scores obtained when Gm positive and negative reference sera were tested for the different Gm characters. They distributed within two clearly separated ranges of lower and higher agglutination scores, respectively. Similar distributions were found also for myeloma sera with a very low content of normal γ globulin, for isolated M components, and for normal γ-globulins. Sera containing M components and larger amounts of normal γ globulin frequently gave intermediate reactions in the Gm(b) system, and sometimes also in the Gm(a) and the Gm(x) systems.

Some sera and isolated proteins deviated from the usually encountered reaction pattern. Three unseparated sera were found to contain an agglutinating factor directed to cells sensitized with Rh antibody used in the Gm(a) and the Gm(x) test systems. As reliable grouping could not be established, these sera are not included in the corresponding histograms. Any possible changes in Gm type or inhibiting titre due to partial denaturation of protein, or due to the presence of aggragated γ-globulin, were carefully controlled and excluded.

Table 1 demonstrates the frequency of the Gm characters of the isolated M components and normal γ globulin preparations and compares these with the data on normal Scandinavian sera (*Harboe & Lundewall* 1959, *Harboe* 1959a). Normal sera and the normal γ-globulin from the pathological sera resemble each other [illegible] inct- [illegible] the [illegible] pos- [illegible] characters they were either Gm(a+x+b—), Gm(a+x—b—) or Gm(a—x—b+). The combination Gm(a+b+) as commonly encountered in normal γ globulin, was never observed. This

Paper electrophoresis was performed according to *Laurell et al* (1956)

Starch gel electrophoresis was performed according to *Smithies* (1955) using the original horizontal method and a borate buffer of pH 8 4

Immunoelectrophoresis was performed according to *Scheidegger* (1955) with some modifications (*Heremans* 1960) The patterns were developed with a variety of anti sera which were produced by immunizing rabbits with purified 7 S γ globulin obtained from pooled sera and with pooled isolated M components of the β_M or the β_A type High titered sera were selected pooled and rendered specific to either 7 S γ β_M or β_A globulin by appropriate adsorption

Isolation of 7 S γ globulin and M components The procedure as outlined by *Laurell* (1961b) was usually followed Crude γ globulin fractions were obtained from sera by precipitation at 40 per cent ammonium sulfate saturation In those instances where the serum contained virtually no normal γ globulin the M-component was sometimes isolated by the modified rivanol method (*Horejsi* & *Smetana* 1956) In all other instances isolation of the M-components and of the normal γ globulin was accomplished by a combination of methods

Chromatography was performed according to *Sober* & *Peterson* (1958) The protein was eluted first with starting buffer and then by means of a pH and ionic strength gradient produced in an open cone sphere system TRIS (hydroxyaminomethane) phosphate buffer of pH 8 6 0 005 M and of of pH 6 0 0 3 M was used as starting and limiting buffer, respectively The fractions obtained concentrated by ammonium sulfate precipitation or ultrafiltration were analyzed with paper electrophoresis and compared with the original serum Starch gel and immunoelectrophoresis were used to determine their degree of purity

Preparative zone electrophoresis in Pevikon blocks according to *Muller Eberhard* (1960) was used for the separation of two M components in one serum

Ultracentrifugation was performed in a Spinco model L ultracentrifuge Density gradients were employed to verify molecular homogeneity of 7 S material (*Kunkel* 1960) To eliminate aggregates some 7 S γ globulin fractions were centrifuged at 40 000 rpm in a 40 2 rotor for 3 hours 5 5 ml of a 1 per cent solution were used and the top 1 5 ml recovered after centrifugation

Serological techniques Buffered saline (3 parts of 0 85 per cent saline + 1 part phosphate buffer pH 7 2 M/15 according to Sorensen) was used for all serological analyses

Gm tests were performed on solutions adjusted to equal γ globulin concentration Isolated fractions were determined by the Biuret method The γ globulin content of unfractionated sera was ascertained by paper electrophoresis and from the total serum protein When isolated γ globulin fractions were measured Cohn Fr II γ globulin was used as standard for the Biuret method When total protein of sera was determined albumin was used as standard γ globulin concentrations determined by paper electrophoresis were multiplied by a factor 1 4 to account for the under estimation by the method (cf *Laurell* 1961a) In some instances when only very small amounts of normal γ globulin could be isolated the concentration was evaluated from the UV absorption at 210 mμ (*Tombs et al* 1959) using γ globulin as standard

The Gm typing was performed according to *Harboe* (1959b) The capacity to inhibit agglutination was measured in two ways Constant amounts of γ globulin were reacted with six doubling dilutions of the rheumatoid serum[1] and constant amounts of Rh sensitized cells Agglutination was scored from 0 to 4 and the sum of all scores was taken as one measure of inhibition capacity Or a series of doubling dilutions of the inhibiting preparation (isolated myeloma protein isolated normal γ globulin or γ globulin in whole serum) was added to constant amounts of rheumatoid serum and sensitized cells The inhibition titre was defined as the highest dilution giving complete inhibition Appropriate controls were always run Throughout the experiments identical dilution ranges of rheumatoid sera and sensitized Rh Rh₂ red cells in 0 5 per cent suspension were used The reactants were added in 0 04 ml aliquots with calibrated Pasteur pipettes Agglutination inhibition titres were only compared when the tests were performed with identical batches of reagents

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1 Rheumatoid anti Gm(a) and anti Gm(b) sera kindly furnished by Prof *R Grubb* and Dr *L Mårtensson*

is illustrated in Table 2, which demonstrates the correlation between the Gm types of M-components and corresponding normal γ globulin. The Gm character of an M component was always found in the normal γ globulin from the same serum. M components lacking any Gm characters tested for were found in six out of nine sera of Gm(a−x−b+) normal γ globulin and only in two out of eleven cases of Gm(a+x+b+), Gm(a+x+b−), or Gm(a+x−b−) normal γ globulin.

TABLE 1

The Frequency of Gm Characters in Normal Sera[1] in Isolated Residual Normal 7 S γ Globulin From Sera with M Components and in 7 S γ M Components

	Gm (a)	Gm (x)	Gm (b)
Normal sera (%)	61	26	82
Normal γ globulins (%)	55	30	70
(Total No. 20)	(11)	(6)	(14)
M Components (%)	47	17	8
(Total No. 36)	(17)	(6)	(3)

[1] According to *Harboe & Lundewall* (1959) and *Harboe* (1959a)

TABLE 2

Gm Types of Normal 7 S γ Globulins and Corresponding 7 S γ M Components

Normal γ Globulins	M Components					
	Gm (a+x+b+)	Gm (a+x+b−)	Gm (a+x−b−)	Gm (a+x−b+)	Gm (a−x−b+)	Gm (a−x−b−)
Gm(a+x+b+)	0	2	0	0	0	1
Gm(a+x+b−)	0	2	0	0	0	1
Gm(a+x−b−)	0	0	2	0	0	0
Gm(a+x−b+)	0	0	3	0	0	0
Gm(a−x−b+)	0	0	0	0	3	6
Gm type unknown	0	2	6	0	0	8*
Total	0	6	11	0	3	16

* Two Gm(−) M Components were present in one of the sera investigated

Repeated titrations revealed sixteen times higher agglutination inhibition in the Gm(b) system of two Gm(a−x−b+) M components as compared to normal Gm(a+x+b+), Gm(a+x−b+), or Gm (a−x−b+) γ-globulin. In the Gm(a) system two Gm(a+x+b−) and three Gm(a+x−b−) M-components showed equal, or two to four

---

Figs. 1–3

Histograms showing the distribution of agglutination scores in Gm typing of

A. Normal Gm positive and negative reference sera
B. Sera containing an M-component but virtually no normal γ globulin
C. Sera containing an M component and varying amounts of normal γ globulin
D. Isolated M components
E. Isolated normal γ globulin from sera containing an M component

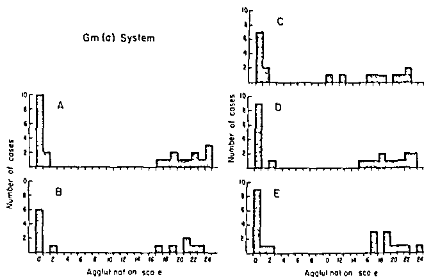


Fig 1

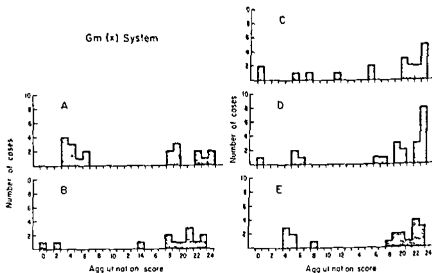


Fig 2

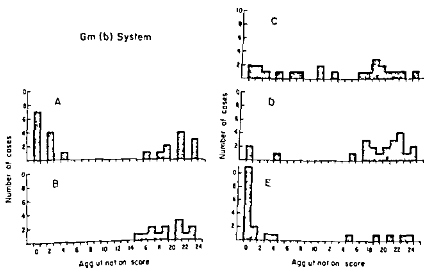


Fig 3

plasma cells where each single cell produces γ globulin of the type Gm(a+x+b−), Gm(a+x−b−), Gm(a−x−b+), or Gm(a−x−b−), and where, furthermore, the proportion of molecules of a certain genetic type directly reflects the proportion of the corresponding synthesizing cells. Random mutation leading to the development of an M component of a certain Gm type, would appear in a frequency determined by the proportion of the corresponding cells among the whole 7 S γ globulin synthesizing plasma cell mass. In the following these proportions will be calculated on the basis of the frequency of the Gm characters in M components studied by the present author and by *Mårtensson* (1961) and *Harboe et al.* (1962b) (Table 3).

TABLE 3

Gm Types of Normal 7 S γ Globulins and of Corresponding 7 S γ M Components Accumulated Material from the Present, from Mårtensson's (1961) and from Harboe et al. (1962 b) Investigations

	Normal γ-Globulin	M Components			
		Gm (a+b−)	Gm (a+b+)	Gm (a−b+)	Gm (a−b−)
Mårtensson (1961)	Gm type unknown	7	0	3*	14§
Harboe et al. (1962b)	Gm(a+b−)	2	0	0	3
	Gm(a+b+)	11	0	1	3
	Gm(a−b+)	0	0	1	14
	Gm type unknown	0	0	0	5
Own material	Gm(a+b−)	4	0	0	1
	Gm(a+b+)	5	0	0	1
	Gm(a−b+)	0	0	3	6
	Gm type unknown	8	0	0	8
Total		37	0	8	55

* Includes one M Components grouped as Gm(a±b+) by *Mårtensson*

§ Includes five M-Components grouped as Gm(a±b−) by *Mårtensson*

Considering the differences in frequency of the Gm characters in American white and Scandinavian population th> Cm(a) and the Gm ...

... *Steinberg et al.* 1960). The Gm(x) character was expected in 26 per cent (*Harboe & Lundevall* 1959). The finding of 37 Gm(a+) out of 100 M components suggests that approximately 62 per cent of the total plasma cell population producing 7 S globulin ... Gm(a+) ...

... the basis of our own material is 64 per cent, which is consistent with the idea that the Gm(a) and Gm(x) characters may be present on the same molecules or synthesized by the same cells. The fraction of cells producing Gm(b+) γ globulin appears to be 10

times greater agglutination inhibition activity than Gm(a+x+b—), Gm(a+x+b+), or Gm(a+x—b+) normal γ-globulin No differences were observed for the inhibition activity in the Gm(x) system, when the M-components were compared with Gm(a+x+b+) or Gm (a+x+b—) normal γ-globulins

DISCUSSION

Gm typing of normal γ-globulin and isolated M-components in this study agrees with that presented in other investigations (*Mårtensson* 1961, *Harboe et al* 1962b) The M-components were found to be either Gm(a+x+b—), Gm(a+x—b—), Gm(a—x—b+) or Gm(a—x—b—) In no instances were both the Gm(a) and the Gm(b) characters found in a single component Six M-components were grouped as Gm (a+x+b—), supporting the contentions of *Mårtensson* (1961) and *Harboe et al* (1962b) that these characters may be present on the same molecules In twenty cases it was possible to compare the isolated M-components and normal γ-globulin from the same sera The Gm character, if present on an M-component, was always found in the corresponding normal γ-globulin The Gm characters of the normal γ-globulin preparations did not show any gross deviations from those of normal Scandinavian sera, indicating no correlation between Gm phenotype and the development of an M-component

Harboe (1960) and *Mårtensson* (1962) studied the agglutinability of Rh sensitized cells by different anti Gm sera Their findings suggest a Gm specificity of the Rh antibody very similar to that found among M-components Not infrequently, completely non-reacting cells were found, or cells reacting with agglutinators directed towards one of the Gm characters demonstrable in the corresponding whole anti-Rh serum Also, the frequency of the Gm characters of the antibodies seemed to parallel that found among M-components These similarities suggest that myeloma proteins correspond to normal molecules of γ-globulin in respect to Gm properties

It was found in the present study, in accordance with *Mårtensson* (1961) and *Harboe et al* (1962b), that Gm(b+) M components are more potent agglutination inhibitors than normal Gm(b+) γ globulin This would be expected if an M component of high molecular homogeneity is compared with normal γ globulin of heterogeneous composition The Gm(b) character obviously is carried only by a small proportion of molecules of normal γ-globulin The Gm(a) and the Gm(x) characters seem to be present on a greater proportion of molecules as the titrable difference was considerably less expressed for these characters

There is an apparent correlation between the indicated frequency of the genetic characters among the molecules of normal γ-globulin and their appearance among M-components This is compatible with a concept that 7 S γ-globulin is synthesized by a heterogeneous population of

plasma cells where each single cell produces γ-globulin of the type Gm(a+x+b—), Gm(a+x—b—), Gm(a—x—b+), or Gm(a—x—b—),

of a certain Gm type, would appear in a frequency determined by the proportion of the corresponding cells among the whole 7 S γ-globulin synthesizing plasma cell mass In the following, these proportions will be calculated on the basis of the frequency of the Gm characters in M components studied by the present author and by *Mårtensson* (1961) and *Harboe et al* (1962b) (Table 3)

TABLE 3

Gm Types of Normal 7 S γ Globulins and of Corresponding 7 S γ M Components Accumulated Material from the Present from Mårtensson's (1961) and from Harboe et al (1962 b) Investigations

	Normal γ-Globulin	M Components			
		Gm (a+b—)	Gm (a+b+)	Gm (a—b+)	Gm (a—b—)
Mårtensson (1961)	Gm type unknown	7	0	3*	14§
Harboe et al (1962b)	Gm(a+b—)	2	0	0	3
	Gm(a+b+)	11	0	1	3
	Gm(a—b+)	0	0	1	14
	Gm type unknown	0	0	0	5
Own material	Gm(a+b—)	4	0	0	1
	Gm(a+b+)	5	0	0	2
	Gm(a—b+)	0	0	3	6
	Gm type unknown	8	0	0	8
Total		37	0	8	55

* Includes one M Components grouped as Gm(a+b+) by *Martensson*
§ Includes five M Components grouped as Gm(a+b—) by *Martensson*

Considering the differences in frequency of the Gm characters in American white and Scandinavian population, the Gm(a) and the Gm(b) characters were expected in 60 and 86 per cent, respectively, of the normal γ globulins of the accumulated cases (*Harboe & Lundevall* 1959, *Harboe* 1959a, *Steinberg et al* 1960) The Gm(x) character was

ıg of 37

62 per

[illegible] plasma cell population producing 7 S γ globulin in a Gm(a+) individual is concerned with the synthesis of molecules of this particular Gm type The proportion of plasma cells producing Gm(x+) γ globulin, calculated on the basis of our own material, is 64 per cent, which is consistent with the idea that the Gm(a) and Gm(x) characters may be present on the same molecules or synthesized by the same cells The fraction of cells producing Gm(b+) γ-globulin appears to be 10

per cent It follows that the proportion of plasma cells synthesizing Gm(—) molecules is 38 per cent in Gm(a+b—), 28 per cent in Gm (a+b+) and 90 per cent in Gm(a—b+) individuals

The allotype system of rabbits has many features in common with the genetic system of human γ-globulin *Oudin* (1960) noticed individual variations in the concentration of the allotypes, which are explained by the possible dosage effect in hetero- and homozygous individuals If the proportion of molecules carrying a Gm character varied similarly, this might be reflected in the frequency at which the character appears in M-components of genotypically different subjects In the accumulated material, a variation to an extent as observed by *Oudin* should be noticeable, at least for the Gm(a) character This was not the case, however, as six of the Gm(a+) M-components were derived from the homozygous and sixteen from the heterozygous individuals Assuming that 62 per cent of plasma cells are synthesizing γ-globulin of this type in both homo- and heterozygous individuals, the calculated numbers of Gm(a+) M-components of each respective group are six and thirteen

SUMMARY

The Gm types of M-components from 35 Scandinavian sera were investigated In 20 cases the corresponding residual normal γ-globulins were isolated for comparative serological analysis The Gm(a) and the Gm(x), the Gm(a) or the Gm(b) factors were found in single 7 S γ-globulin M components No component contained both the Gm(a) and the Gm(b) factors, though both of these in several instances were demonstrated in the corresponding normal γ-globulin Many of the components lacked these genetic factors The Gm serology of the isolated normal γ-globulins appeared similar to that of normal Scandinavian sera The possible normal molecular and cellular distribution of the Gm characters as indicated by their frequency among M-components is discussed

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The Institute of Forensic Medicine, University of Lund Sweden

EFFECT OF MODE OF SACRIFICE ON HISTOCHEMICALLY DEMONSTRABLE ACTIVITY OF UNSPECIFIC ESTERASES AND UNSPECIFIC ALKALINE MONOPHOSPHATASES IN GOLDEN HAMSTERS

By

GORAN SKOLD and ULF BRUNK

Received 19 xi 63

Recent years have witnessed a surge of interest in the histochemically demonstrable activity of different enzymes and their distribution under various experimental conditions The purpose of the present investigation was to find out whether the mode of sacrifice of the experimental animals had any effect on the histochemically demonstrable activity and distribution of unspecific esterases and unspecific alkaline monophosphatases

MATERIAL AND METHODS

The material consisted of 30 male hamsters aged three to four months and belonging to the stock of the institution The animals had been brought up on a uniform diet The series was divided into six equal groups and each group was sacrificed in a different way

The methods used were as follows

1 *Decapitation*

2 *Poisoning with hydrogen sulphide* The animals were placed in a glass container into which hydrogen sulphide gas was conducted The animals were removed immediately after respiratory movements had ceased usually after about 35 40 seconds

3 *Poisoning with chloroform* The animals were placed in a glass container with a ball of cotton wool soaked in chloroform The animals were removed as soon as respiratory movements had ceased i e after 3 5 minutes

4 *Poisoning with ether* The animals were placed in a glass container with a ball of cotton wool soaked in ether The animals were removed as soon as respiratory movements had ceased i e after 3 5 minutes

5 *Poisoning with coal gas* The animals were placed in a glass container into which gas from the mains was conducted They were removed as soon as respiratory movements ceased ie after 3 5 minutes

6 *Poisoning with Nembutal®* A dose of 20 mg was injected intraperitoneally and the animals died 7 12 minutes later

The animals were examined immediately after they had died and pieces of the liver, adrenals kidney small intestine cerebrum and heart were removed for histochemical examination The samples were cut into thin slices at most 3 mm thick The preparations were placed immediately in 10 per cent neutral formalin at +4° C buffered with 0 1 M citrate buffer pH 7 0 The samples were fixed at +4° C for 16 hours

Frozen sections 10 µ thick were mounted on clean slides and air dried for 6 hours at room temperature

According to the instructions given by *Pearse* one to three hours is a suitable
time for air drying the preparations before staining In our investigation however
we found prolongation of this time to 6 hours to result in much better pictures for
demonstrating alkaline phosphatases in the small intestine We feel that artefacts
due to diffusion are considerably reduced by this procedure Prolongation of the
drying period of the preparations from the other organs studied had no such effect
Unspecific alkaline monophosphatases were demonstrated by *Crogg* & *Pearse* s

FINDINGS

Cerebrum Unspecific esterases were demonstrated in the cytoplasm of the pyramidal cells and to a smaller extent in that of other large nerve cells The glia cells and capillary walls did not stain Nowhere in the brain did the nuclei take on the stain

Unspecific alkaline monophosphatases were demonstrated in the ependyma in the choroid plexus and in the capillary walls No alkaline phosphatase was found in the nerve cells

Heart The heart muscle cells showed no unspecific esterases

Abundant unspecific alkaline monophosphatases were found in the capillary walls of the heart No enzyme could be demonstrated in muscle cells

Liver The liver cells proved rich in unspecific esterases which were evenly distributed in the cytoplasm The nucleus was void of esterase Within the lobular unit the activity tended to be slightly higher around the central veins The epithelium of the bile ducts also stained

No unspecific alkaline monophosphatases were found in the actual liver cells On the other hand the enzymes were demonstrated in the cytoplasm of the leucocytes in the sinusoids

Adrenals Small amounts of esterase were demonstrated in the apexes of the glandular cells in some areas of the medulla The cortex contained small amounts of enzyme in the zona glomerulosa and in the outer region of the zona fasciculata Only the cytoplasm took on the stain

No alkaline phosphatases were demonstrated in the medulla In the cortex the enzymes were situated mainly in the inner parts of the zona reticularis and in the inner parts of the zona fasciculata The zona glomerulosa also contained some enzymes while the outer part of the zona fasciculata was broadly speaking void of enzyme The enzymes were situated mainly in the marginal part of the cytoplasm while the nucleus never stained anywhere in sections of [illegible]

in

hi [illegible] of the cells The ganglion cells in Auerbach s plexus stained only slightly Nowhere in the small intestine did the cell nuclei take on the stain

Also alkaline phosphatases were demonstrated only in the cytoplasm

Acta path et microbiol scandinav 61, 190–194, 1964

The Institute of Forensic Medicine, University of Lund Sweden

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Recent years have witnessed a surge of interest in the histochemically demonstrable activity of different enzymes and their distribution under various experimental conditions The purpose of the present investigation was to find out whether the mode of sacrifice of the experimental animals had any effect on the histochemically demonstrable activity and distribution of unspecific esterases and unspecific alkaline monophosphatases

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Frozen sections 10 µ thick were mounted on clean slides and air dried for 6 hours at room temperature

The enzymes were distributed in a thick band immediately beneath the cuticular border and in a thinner layer immediately apical to the basal cell nuclei

Kidneys The proximal convoluted tubules contained abundant esterases while the other parts of the tubules stained only weakly In the proximal collecting tubes every third to fourth cell contained small apical collections of granulae The cell nuclei were unstained

Abundant alkaline phosphatases were found in the proximal convoluted tubules The enzymes were found in the apical parts of the cells The cell nuclei did not stain No alkaline phosphatases were demonstrable within the glomeruli other parts of the tubules or the collecting tubes

The site and intensity of the enzymes in the preparations was found not to vary with the way the animals were killed

DISCUSSION

In all investigations of enzymes it is of course necessary to ascertain whether and if so to what extent handling or treatment of the material affects the activity or distribution of the enzymes Most publications bearing on this problem however deal with the decrease in activity in association with the different steps of the histological preparation of the specimens (1 2 3 7 8) The effect of autolysis has also received attention (4) But a search of the literature failed to reveal more than one investigation touching upon the effect of the way in which the animals were sacrificed namely that by *Sachs et al* (6) who found that the activity of the alkaline phosphatases in the kidneys in rats and cats did not vary ... studied acute poisoning ... ide methyl alcohol carb ... that no investigation ... exclusively with this problem has ever been published

All investigators seem to have taken for granted that the way in which the animals are sacrificed has no substantial influence on the activity and distribution of enzymes demonstrated histochemically Though this problem is of minor significance in an investigation in which all the animals are killed in the same way it should nevertheless be borne in mind when comparing the res...

... by the stress to which the animal is subjected

Judging from the findings in the present investigation however neither the activity nor the distribution of unspecific alkaline monophosphatases and unspecific esterases in the liver kidney adrenal

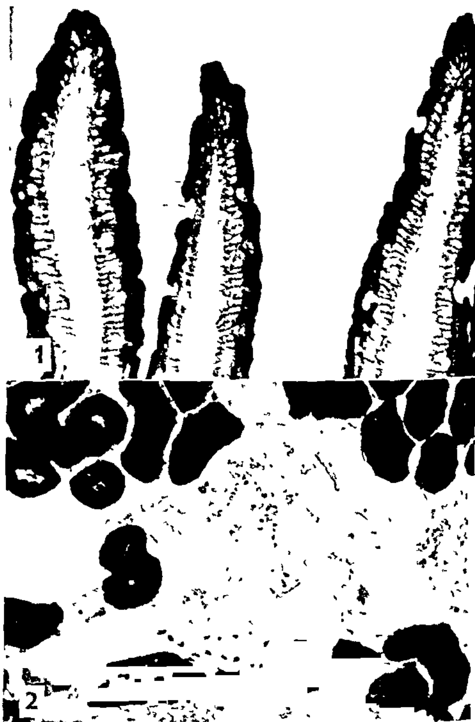


Fig. 1. Activity of unspecific alkaline monophosphatases in small intestine
Fig. 2. Activity of unspecific esterases in kidney

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The Inst tute of General Pathology University of Aarhus (Head Professor J B chel M D) and The Radium Centre Aarhus (Head Professor S haae M D)

A SPONTANEOUS TRANSPLANTABLE C_3H MOUSE LEUCOSIS (NJA)[1]

By

KAJ OLSEN

Received 4 x 63

Among other features of the inbred C3H mouse strain, it should be mentioned that this strain often develops spontaneous mammary cancer whereas spontaneous occurrence of leucosis is rare In October 1960 the NJA leucosis appeared in a 81 days old untreated female mouse and appeared to be well suited for cancer chemotherapeutic and cancer biological research (*Bach Bichel & Hejgaard* 1963 *Olsen* 1963) Hence the present paper describes some of its characteristic biological qualities

MATERIAL AND METHODS

The C3H Mice

The C3H mouse stra n has been ma nta ned by brother sister inbreeding in the Rad um Centre in Aarhus since 1950 (*Nielsen* 1962) and also in the Institute of General Pathology at the Un versity of Aarhus

Techn que of Transplantat on

The d nor mice are k lled by a pull at the n k Th

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H stolog cal Descr ption

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ther tissues w gans and
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[1] Named after our laboratory superintendent *Niels Jegstrup* Aarhus
Aided by grants fr m the Anders Hasselbalch Anti Leucaem Foundation and *Carl Schepler and Wife s Bequest* the *Irma Foundation*

heart, cerebrum and small intestine in golden hamsters varies with the way the animals are sacrificed

SUMMARY

Histochemically demonstrable unspecific esterases and unspecific alkaline monophosphatases in the liver, kidneys, adrenals, small intestine, heart and cerebrum in golden hamsters were studied for any variation in the distribution or activity of the enzymes in preparations with the way in which the experimental animals were killed No such variation was found in animals killed by decapitation, or poisoned with hydrogen sulphid, ether, chloroform, coal gas or Nembutal®

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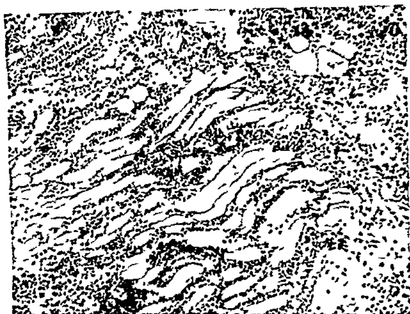


Fig 1b
Subcutane us NJA tumour 100 ×



Fig 1c
NJA infiltration in liver 100 ×

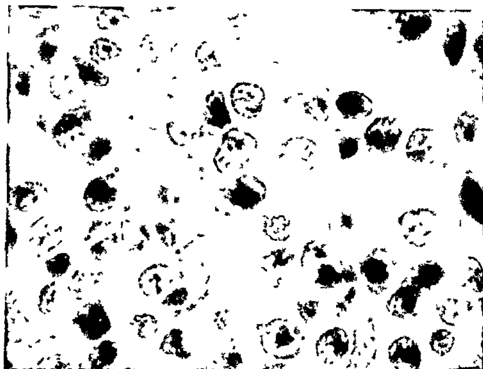


Fig 1a
NJA tumour 400 ×

Fig 1
Haematoxylin eosin stain Thickness of section 5 μ

nucleus is rounded or kidney shaped with a high content of chromatin which is often arranged like a network Appearance of nucleoles is not a constant phenomenon but they appear sporadically in numbers of two or three at a time in the same cell The cytoplasm is scarce without granules and stains neutrophilically or slightly basophilically with haematoxylin eosin

In smears of the bone marrow from experimental animals with advanced NJA leucosis the erythropoietic tissue is often displaced completely by malignant cells which in this case resemble the tumour cells of the local tumour However as is the case in the peripheral blood the tumour cells in the bone marrow are very fragile and are often damaged during the preparation of the specimen Many degenerated tumour cells without visible nuclear membrane are seen and in such cells the distribution of the chromatin is often of an even more net like appearance The diameter of such cells approaches often 40 μ

The heavily enlarged spleen is dominated by malignant cells and even in moderately advanced cases the structure is completely obliterated However capsula and trabeculae are preserved to a wide extent

In the liver the leukaemic infiltration appears first around the portal vessels where as it were a jacket of malignant cells grows up (Fig 1 c) In more advanced cases the liver tissue is diffusely infiltrated however so that the coarse structure of the liver is preserved

The fatty capsula and the hilus regions of the kidneys show massive infiltration of leukaemic cells at an early stage and the kidney itself is affected most markedly in the cortical zone from where the infiltration abates towards the pyramids Often the pathological lesions seem to be most pronounced in radial segments of the kidney

Just as the splen the thymus and the lymph nodes are quite pervaded by leukaemic tumour masses and lose their original structure completely The adrenals are infiltrated most markedly in the peripheral regions but in rare cases only beyond the cortical zone

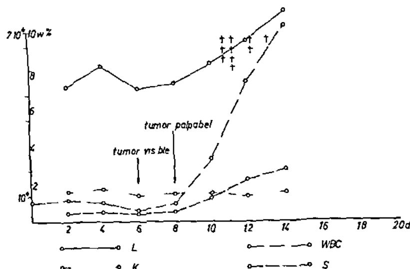


Fig 2

Development of the NJA leucosis

40 mice were inoculated subcutaneously in the right axilla at time zero with 10^6 NJA tumour cells in 1 ml physiological saline solution Each second day 4 animals were killed Leucocyte count (WBC) and weight per cent of spleen (S) liver (L) and kidneys (K) were plotted as a mean of the 4 values

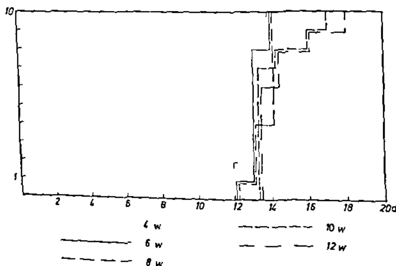


Fig 3

Age of the recipient mice

5 groups of mice were selected of the ages 4 6 8 10 and 12 weeks (w) respectively at the day when all of them were inoculated with 10^6 NJA tumour cells per mouse

The lung tissue is fairly constantly free from the leukaemic infiltration but from the hilus streaks of solid leukaemic tissue projects between the lung lobes

Apart from the site of the local tumour, no leukaemic infiltration is found in the muscles or the brain tissue, not even after intracerebral inoculation but malignant cells are present in great numbers in blod vessels and terminally also around the vessels

In advanced cases smears of peripheral blood contain a large number of leukaemic cells the cytoplasm of which is often heavily basophile with Giemsa stain *Immature erythrocytes and reticulocytes with pronounced basophilia and often* remnants of nuclei are also constant findings

RESULTS

After subcutaneous inoculation with 10^6 NJA tumour cells, a local tumour develops on about the 6th to 7th day, and typical blast cells appear in the peripheral blood The "white blood count" increases from normal values of 5 to 10 thousand to more than 100,000/µl At the same time the weight of the spleen increases from 6 to 8 times and the weight of the liver by about 50 per cent Death occurs at about the 13th day (Figs 2 and 13) Transplantation with peripheral blood drawn after the 8th day was carried out in passage over more than one year

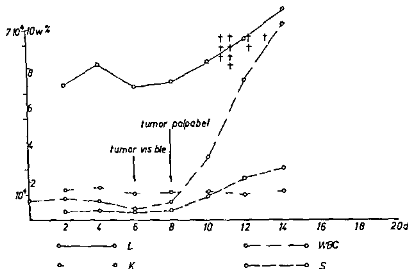
Survival curves for 5 groups of mice show no variations in the course of disease (Fig 3)

NJA tumour cells isolated between the 9th and the 14th day after inoculation of the donor animals were transferred to 4 groups of mice, the survival period of which seems to be independent of the age of the inoculum within this interval (Fig 3)

In a titration experiment, groups of mice were inoculated with a ten fold dilution series of the same suspension of NJA tumour cells (Fig 5) This experiment and similar titration experiments (Fig 15) contributed to the evaluation of a standard inoculum of 10^6 NJA tumour cells The inaccuracy involved in the counting of tumour cells has been estimated at 10 to 20 per cent (evaluated on the basis of repeated countings on the same sample and after the drawing of various samples of the same suspension of tumour cells The countings were performed by different trained persons) Furthermore, the evaluation of the vitality of the tumour tissue at the time when it is removed for transplantation is encumbered with uncertainty The standard inoculum chosen is so high that in practice it is possible to exclude unsuccessful inoculations on account *of technical errors*

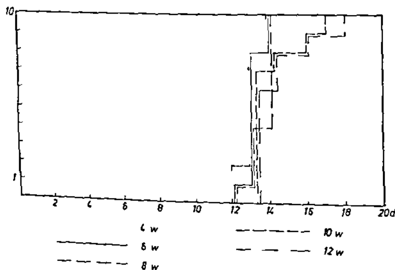
Fig 15 indicates that the susceptibility of the C_3H mice with regard to intracerebral inoculation is not essentially different from the susceptibility after subcutaneous inoculation

After intraperitoneal inoculation, the C_3H mice develop universal NJA leucosis without local tumour In some cases, but not always, moderately enlarged lymph nodes may be found in the mesentery Even after repeated intraperitoneal inoculations with 10^7 NJA tumour cells, no ascites tumour developed The survival periods following intraperitoneal and subcutaneous *inoculation are comparable*



F g 2
Development of the NJA leucosis

w th 10⁶
animals
(L) and



F g 3
Age of the recipient m ce
5 groups of m ce were selected of the ages 4 6 8 10 an l 12 weeks (w) respect vely at the day when all f them were inoculated with 10⁶ NJA tumour cells per mouse

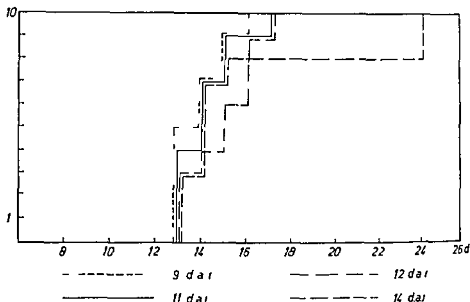


Fig 4

Age of NJA tumour

9 11 12 and 14 days after simultaneous subcutaneous inoculation with the same dose into donor mice NJA tumour cell suspensions in physiological saline solution were prepared Out of each suspensions 10 mice were inoculated with 10^4 NJA tumour cells per mouse

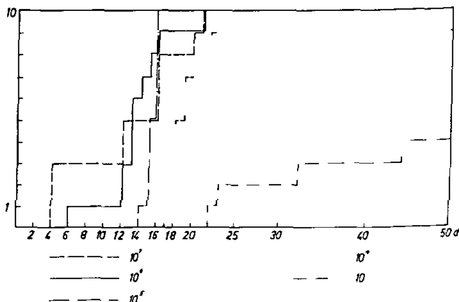


Fig 5

At time zero groups of 10 mice were inoculated with 10 tumour cells and logarithmically decreasing doses Three mice died on the 4th day without signs of NJA leucosis

Fig 13 presents the survival periods for 10 control animals from each of 53 experiments Two mice were rejected viz one mouse which died on the 3rd day from unknown cause and another one which survived the 50 days of observation of the experimental period without any signs

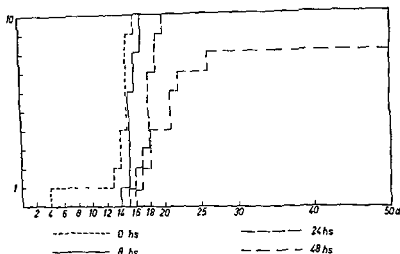


Fig 6

Stability of NJA at 4° C

At time zero 8 24 48 and 120 hours 10 mice from each group were inoculated with 10^6 NJA tumour cells Even after 120 hours storage at 4° C no loss of cells through clinging to the glass Take and time of survival after oculation were fairly constant over 8 hours with the employed inoculum 24 hours inoculation still resulted in a 100 per cent mortality, while 2/10 mice survived the inoculation in the "48 hours group" At inoculation at time 120 hours no development of tumour was seen One mouse died on the 4th day without NJA leucosis

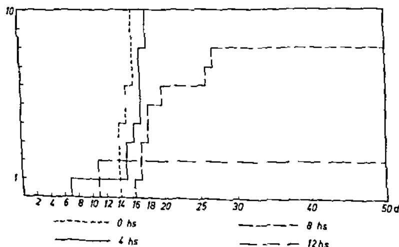


Fig 7

Stability of NJA at 20° C

At time zero 4 8 12 and 24 hours 10 mice were inoculated with 10^6 NJA tumour cells 2/10 mice survived inoculation with tumour cells which had been incubated at 20° C over 4 hours Neither local tumour nor leukaemia had developed in the 12 hours and the 24 hours groups The deaths recorded on the 7th and 11th day were not caused by NJA leucosis

of tumour development On the 81st day after the first inoculation, the latter animal was again inoculated subcutaneously with 5 by 10 NJA tumour cells and now it developed a typical NJA leucosis parallel with 10 control mice of a new experimental series It died on the 14th day after the second inoculation Resistance to tumour thus being excluded, it is most likely that this mouse was not inoculated the first time

From the separate survival curves for male and female mice it appears that the male mice survive about 10 per cent longer than the female mice

In other respects the NJA tumour fulfills the requirements to a transplantable tumour which are essential for this category of biological experiments, and of which the most important ones are a high mortality and limited dispersion of the survival time,

on the 13th and 14th day after inoculation 280/530 mice or 53 per cent die,

on the 12th to 15th day after inoculation 434/530 mice or 82 per cent die,

TABLE 1

Survival of Control Animals after Inoculation with 10 NJA Tumour Cells

Day of death	♂	♀	Total
9	1	2	3
10	1	2	3
11	2	16	18
12	16	62	78
13	59	98	157
14	82	41	123
15	53	23	76
16	32	10	42
17	8	5	13
18	7		7
20	1		1
21	5		5
2[illegible]	1	1	2
	268	260	528
Mean	14 4	13 1	13 8 days

In order to elucidate how fast the NJA leucosis becomes generalized, spleen tissue was transplanted after the expiration of different periods after subcutaneous inoculation with NJA tumour (Fig 11) 120 donor mice were inoculated simultaneously with 10^6 NJA tumour cells of the same suspension and from the following day 10 mice are killed and the spleen removed One third of each spleen is reserved for histological examination, while the remaining part is comminuted by cutting and mixed, whereupon this material is inoculated subcutaneously into 10 new mice

The first, second, and third groups of experimental mice remained unaffected by the spleen transplantation From the 4th and 5th days

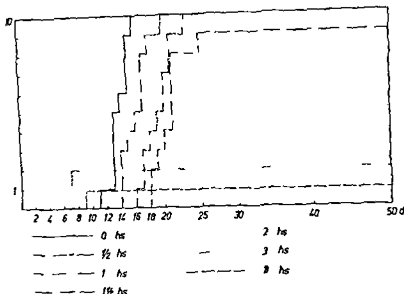


Fig 8

Stab l ty of NJA at 37° C

Inoculat on of 10⁶ NJA tumour cells per mouse was performed after incubation of the tumour cells at 37° C in water bath for 0 ½ 1 1½ 2 3 6 8 and 12 h urs As earl as in the ½ hour gr up the recip ent mice exhibited a prol nged period of s r val In the 1½ hour group 1 10 survive At inoculat on at time 3 4 6 8 and 12 hours no cases of development of local tumour or NJA leucosis were observed The deaths re orded on the 4th 7th and 9th days were not caused by NJA leucosis

2 10 and 8 10 mice respectively developed typical NJA leucosis Inocu lation of spleen tissue from donor mice with a more advanced take of tumour shortens the survival time of the recipient mice

Attempts at analyzing the biological activity of the NJA tumour cells over the period from the inoculation to the 4th day have revealed a factor Dispersin (*Olsen* 1963 and unpublished experiments) which seems to participate in the natural resistance of the C3H mice The occurrence of this factor is reflected in the titration experiments (Figs 2 and 1a) and the concentration seems to be most pronounced in the spleen and the pancreas and in the mucous membrane of stomach and intestine of the mice

NJA Autolysis Experiments

The stability of the NJA tumour cells during incubation has been in vestigated in saline suspension at 4°, 20° and 37° C (Figs 6 7 and 8) After incubation on water bath over varying periods 10⁶ NJA tumour cells were at once inoculated into groups of 10 C3H mice for comparison with a control group inoculated with untreated tumour cell suspension

From the survival curves it appears that incomplete mortality is ob

of tumour development. On the 81st day after the first inoculation, the latter animal was again inoculated subcutaneously with 5 by 10^6 NJA *tumour cells and now it developed a typical NJA leucosis parallel with* 10 control mice of a new experimental series. It died on the 14th day after the second inoculation. Resistance to tumour thus being excluded, it is most likely that this mouse was not inoculated the first time.

From the separate survival curves for male and female mice it appears that the male mice survive about 10 per cent longer than the female mice.

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9	1	2	3
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12	16	62	78
13	59	98	157
14	82	41	123
15	53	23	76
16	32	10	42
17	8	5	13
18	7		7
20	1		1
21	5		5
25	1	1	2
	268	260	528
Mean	14.4	13.1	13.8 days

In order to elucidate how fast the NJA leucosis becomes generalized, spleen tissue was transplanted after the expiration of different periods after subcutaneous inoculation with NJA tumour (Fig. 11). 120 donor mice were inoculated simultaneously with 10^6 NJA tumour cells of the same suspension and from the following day 10 mice are killed and the spleen removed. One third of each spleen is reserved for histological examination, while the remaining part is comminuted by cutting and mixed, whereupon this material is inoculated subcutaneously into 10 new mice.

The first, second, and third groups of experimental mice remained unaffected by the spleen transplantation. From the 4th and 5th days

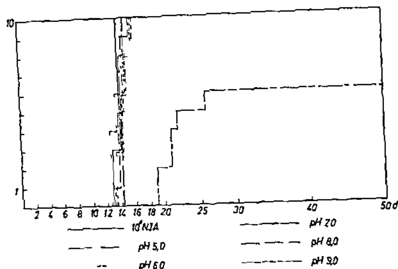


Fig 10

pH stability of the NJA tumour cells

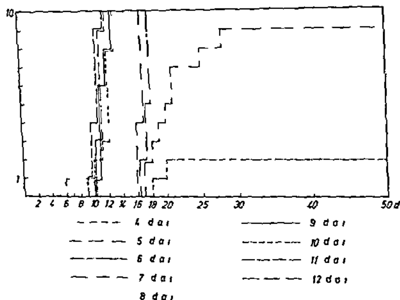


Fig 11

Generalization of the NJA leucosis

The deaths recorded on the 5th and 6th days after inoculating the recipient mice were not caused by NJA leucosis

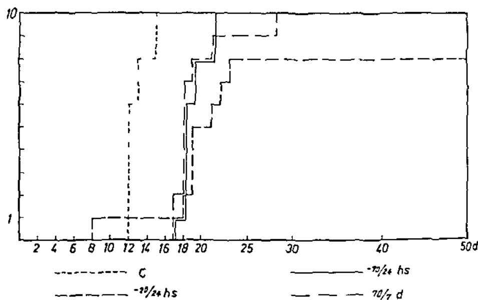


Fig 9
Stability of NJA tumour against freezing thawing

tained after incubation over 48, 8, and 1½ hours, respectively By co parison with the titration curves it appears that this corresponds t maintained ability of take in about 1 per cent of the NJA tumour ce

On the basis of the above, the conclusion was drawn that in prac it is permissible to employ standard inoculation dose for up to 8 ho after isolation at 4° C and for from 3 to 4 hours at 20° C At 37° C, number of tumour cells from an NJA tumour which are capable taking is reduced so rapidly that the time factor is critical

For comparison PBH mammary carcinoma cells (*Nielsen* 1962) h been isolated and incubated at 37° C for 1, 3, and 6 hours as abo All out of 40 C3H mice developed progressive tumour growth upon i culation of 10^6 tumour cells

The stability of NJA tumour tissue during freezing is illustrated Fig 9 The tumour tissue renders material for inoculation of the a mals of the control groups in order to ascertain the initial virulen while the remaining material is frozen in a test tube in dry ice-alcol (at —70° C) for 1 and 7 days without further treatment or admixtu The thawing takes place in a water bath at 37° C for 5 minutes, whe upon a tumour cell suspension is produced as usual Furthermore, mour tissue is frozen at —20° C in an electric freezer and stored at t temperature for 1 and 7 days

The experimental result indicates that under these conditions t NJA tumour tissue maintains a certain ability of taking and apparent is fairly unaffected by the period of storage On the contrary NJA le cosis developed in only 8 10 experimental animals after inoculation wi tumour stored at —20° C for 24 hours and not at all after storage —20° C for 7 days

CO atmosphere) have been exposed to NJA tumour extract, prepared as described above The cultures were examined in vain for such morphological changes as are known from in vitro experiments with polyoma and Rous virus Inoculation of the cells in experimental animals did not induce NJA leucosis

NJA tumour cells could not be cultivated in tissue cultures under the conditions described, but after addition of tumour cells to cultures of normal embryonal mouse cells After inoculation into mice of such mixed cultures NJA leucosis developed after up to 14 days growth *in vitro*

Homologous Transplantation of NJA Leucosis

Street albino mice are resistant to inoculation with NJA tumour cells in doses as high as 3 by 10 /mouse Repeated experiments did neither result in development of a local tumour nor in an increase in the white blood count in the peripheral blood

Ak albino mice (the inbred AKA strain of the Radium Centre) are relatively resistant to transplantation of NJA leucosis In a titration experiment, during which groups of AKA mice were inoculated with 10^4 - 10 - 10 - 10 tumour cells/mouse, a local tumour developed at the usual time Within the group which was inoculated with the highest dose, the tumours reached a size corresponding to that of the C_3H mice between the 8th and the 10th day (about 1 g), but thereafter they regressed and were either resorbed or rejected as necrotic tissue No deaths occurred within the 2 months observation period Following such development of tumour and regression, the AKA mice were resistant to renewed inoculation

In C_3H mice, transplantation of tumour tissue on the 7th and 11th day after inoculation from the AKA mice resulted in a typical NJA leucosis but in new AKA mice no take was observed After regression of tumour in AKA mice, spleen tissue was transplanted on the 27th and 31st days after inoculation, but no take was observed in C_3H or AKA mice

In titration experiments (Fig 12), AKA C_3H F_1 hybrids exhibited a susceptibility, corresponding fairly well to that of the C_3H mice (Fig 5)

Attempts at Active Immunization Against NJA Leucosis

Three groups of C_3H experimental mice, all of which had survived inoculation with NJA tumour cells frozen in saline suspension (cf p 206) were selected for immunization

31 g of NJA tumour tissue was removed from 45 donor mice and homogenized in 180 ml phosphate buffer solution pH 7 2 of physiological ionic strength in a Waring blender and then stored at —20° C in an electric deep-freezer for 160 days 85 days after the above mentioned experimental inoculation, the immunization procedure is com-

In no case the NJA tumour cells which were suspended in physiological saline solution maintained their ability of taking after freezing at —70° C or —20° C and subsequent thawing in a water bath of 37° C for 5 minutes

The pH Stability of the NJA Tumour Cells

The NJA tumour cells are suspended in physiological saline solution adjusted to pH values of 5, 6, 7, 8, and 9 by means of solutions of citric acid and secondary sodium phosphate which is adjusted to physiological ionic strength by adding NaCl:

	A	B
Citric acid	g 7 005	
Sec sodium phosphate	—	g 11 876
Sodium chloride	—7 7	—4 4
Demineralized water up to	1000	1000

After storage for 30 minutes at room temperature, groups of 10 mice are inoculated subcutaneously with 10^6 NJA tumour cells

The survival curves for the experimental mice (Fig 10) make it probable that the NJA tumour cells are unaffected by alterations in pH within the range from 6 to 9 over the period applied At pH 5 so large a proportion of the tumour cells is inactivated that only 6 10 mice die with a prolonged survival period, but indeed with a typical NJA leucosis Necropsy of the surviving mice at the termination of the experimental period reveals no signs of leucosis

Attempts at Cell-Free Inoculation

Cell-free extracts of NJA tumour tissue in physiological saline solution are prepared, the cells being decomposed by freeze-drying (—70° to +37° C), ultrasonic exposure or grinding in a mortar The extract is cleared of remnants of cells by centrifugation (15,000 g for 30 min) and filtration through Millipore filters, pore size 0 45 nm Neither at inoculating 77 mice which were about 6 weeks old (observation period 50 days), nor at inoculating 167 newborn (observed over 2 years) subcutaneously or intracerebrally did we succeed in producing NJA leucosis

Four pregnant mice were inoculated subcutaneously with 10^6 NJA tumour cells and 5 to 7 days later they gave birth to a total of 19 young ones The mothers died 6 to 8 days after delivery, but during this period they had nursed the young ones, which afterwards sucked other C3H mice In no case the young mice developed NJA leucosis (observed over 2 years)

Tissue Culture Experiments

Normal embryonal mouse cells in tissue culture (medium Parker 199 (Difco) with 10 per cent calf serum, grown in Petri dishes in 5 per cent

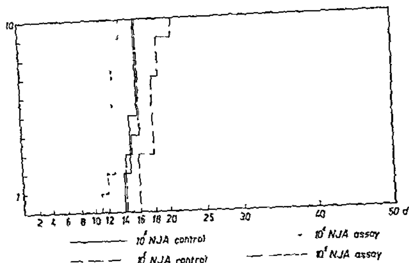


Fig 14

Attempts at immunization against NJA tumour

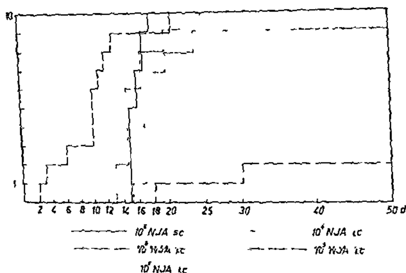


Fig 15

Titration at intracerebral inoculation

The inoculation was performed percutaneously using a Mantoux syringe The NJA tumour cells were suspended in 0 1 ml physiological saline solution No deaths occurred within 50 days in the groups 102 10 and 1 NJA tumour cell per dose The deaths occurring on the 2nd 3rd and 6th days after inoculation were not caused by NJA leucosis

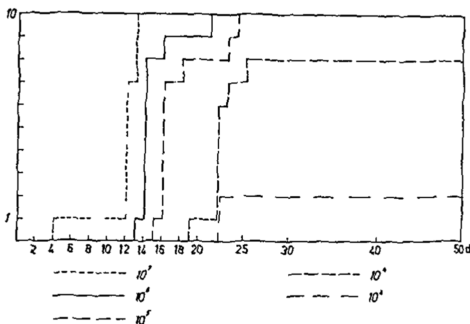


Fig 12
NJA titration in AKA/C_3H F_1 hybrids
This experiment was performed parallel with the experiment presented in Fig 4 the NJA tumour cells being derived from the same isolation
The death recorded on the 4th day was not caused by NJA leucosis

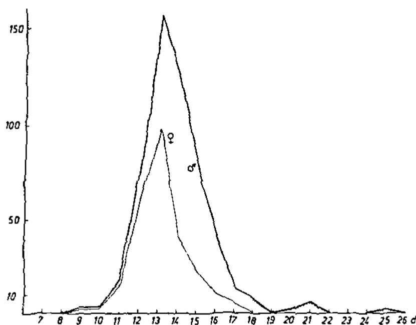


Fig 13
Survival of control animals after inoculation with 10^6 NJA tumour cells

menced by injecting 1 ml tumour suspension mouse twice a week for 3 weeks

1 week after the last injection the experimental group (a) is inoculated with 10^6 NJA tumour cells and group (b) with 10^5 NJA tumour

Acta path et microbiol scandinav 61, 211-232 1964

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THE PARATHYROIDS IN PRIMARY HYPERPARATHYROIDISM

A Histological Study of 35 Cases

By

OLE WERDELIN

Received 8 xi 63

Our present concept of the pathological changes in the parathyroids in primary hyperparathyroidism is largely based on *Castleman & Mallory's* work from 1935 (3) It is now widely accepted that primary hyperparathyroidism may be caused by parathyroid adenomas, by primary hyperplasias, and by carcinomas

The lesion responsible for primary hyperparathyroidism is in most cases a single adenoma in one of the parathyroid glands Double or multiple adenomas do, however, also occur Primary hyperplasia and hypertrophy of the water-clear cell type involving all parathyroid tissue in the patient is the second commonest cause Finally, in a very small number of cases a parathyroid carcinoma is responsible for the hyperparathyroidism

In *Castleman's* (6) 100 cases of primary hyperparathyroidism, adenomas were found in 89 cases, water clear hyperplasia and hypertrophy in 8 and carcinomas in 3 cases

Woolner et al (18) in their 140 cases found 82 1 per cent single adenomas, 4 3 per cent multiple adenomas and 3 6 peer cent multiple tumours of endocrine glands including parathyroids, pancreatic islets and pituitary 8 5 per cent of the cases had primary hyperplasia and hypertrophy of the water-clear type and 1 5 per cent carcinomas

Adenomas

The parathyroid adenoma is a small, rounded, orange-brown, soft mass with a thin capsule The weight and dimensions vary considerably, the largest reported weighing 120 grammees (12) while on the other hand adenomas weighing as little as 100 mg are known to have caused hyperparathyroidism The average size of parathyroid adenomas is about 3 × 2 × 1 5 cm and the corresponding weight seven g

The frequency of occurrence of double and triple adenomas—cases

cells parallel with two control groups Experimental group (c) is observed with a view to possible development of tumours caused by the immunization injections

The survival curves (Fig 14) show the immunization experiment to be negative The injected experimental mice died a little earlier than the corresponding control mice On the basis of this material, any definite conclusion cannot be drawn as to an alteration in the resistance of the experimental mice

SUMMARY

The spontaneous C_3H leucosis (NJA) is a well-suited experimental tumour, the appearance of which is accompanied by characteristic leucaemic blood changes At transplantation 100 per cent mortality is obtained and almost constant survival time in the experimental animals The stability of the tumour cells is studied under varying temperatures and pH conditions They appear to be very sensitive to incubation in physiological saline solution even for brief periods and at the generally employed temperatures

The NAJ leucosis cannot be transferred by cell-free extract, and immunity cannot be induced in C_3H mice

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Nielsen K Udviklingen af metastaser fra et musecarcinom transplanteret på dyrenes haler Universitetsforlaget Aarhus 1962

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very active (18) A rough correspondence between the size of the adenoma and the serum calcium level was found by *Norris* (12) While carcinomas are usually associated with a high serum calcium level and severe osteitis fibrosa primary hyperplasia and hypertrophy do not differ from the adenomas in their clinical aspect (5)

The epithelial cells are most often arranged in solid islands or trabeculae Acinar grouping or cystic pattern occur frequently In some adenomas are seen small or large areas with follicular structure very suggestive of thyroid tissue which may cause confusion at the examination of frozen section preparations

A very characteristic feature of the histological picture is the frequent occurrence of giant nuclei—nuclei several times the normal size Likewise some degree of nuclear polymorphism is not rare The picture may be very bizarre however such features are not indicative of malignancy It may be mentioned that mitoses are extremely rare in adenomas whereas in carcinomas they are usual

The stroma is scanty connective tissue with numerous vessels Sometimes fibrosis calcification or fat is encountered in the stroma

A peculiar finding is the simultaneous occurrence of a parathyroid adenoma and papillary carcinoma of the thyroid Till now 20 such cases have been reported (2 8 13) It is difficult to evaluate the significance of this

Primary Hyperplasia and Hypertrophy

In primary hyperplasia and hypertrophy of the parathyroids all the patient s parathyroid tissue is involved The glands are enlarged with indentations and pseudopod projections on the surface Usually the colour is mahogany brown and much darker than that of the adenoma It is interesting that in contrast to what is the case in adenomas the enlargement of the parathyroids in this condition is usually more pronounced in the upper pair of glands than in the lower Thus *Castleman* & *Cope* (4) found this disproportion in eleven cases the l

Microscopically the parathyroid tissue is composed of water clear cells throughout The cell is greatly enlarged measuring 10–40 μ in diameter and has a quite clear cytoplasm with a well defined cell margin No normal parathyroid tissue is seen outside the capsule *Rogers* & *Keating* (14) by a series of measurements of cell size and volume showed that the condition really is one of hypertrophy and hyperplasia

The nucleus is round and average 6 to 7 μ in diameter There may be some variation in nuclear size but no giant nuclei are seen Although the parathyroid tissue in most cases of primary hyperplasia and hypertrophy consists of water-clear cells throughout cases have however been reported in which areas of smaller cells were encountered (18)

of considerable surgical interest—is stated by *Norris* (12) to be 20 out of his 322 cases collected from the literature (6 2 per cent) *Castleman* (6) found six double adenomas in 100 cases, while *Woolner et al* (18) in their 140 cases found double adenomas in nine and triple adenomas in two cases

Adenomas are more apt to develop in one of the inferior glands than in the superior ones *Norris* (12) thus found adenomas in the lower glands more than four times as often as in the upper glands According to the same author about 10 per cent of all adenomas are found in an aberrant position, most often in the superior mediastinum

Histologically, the adenoma is covered by a thin, well-defined capsule A thin rim of normal parathyroid tissue, characterized by its fatty stroma, is often encountered outside the capsule The occurrence of normal parathyroid tissue outside the adenoma is a reliable criterion that the condition is one of adenoma and not one of hyperplasia, in which case no normal parathyroid tissue is found

The adenomas vary very much both as to the type of cells in them and as to the arrangement of these Microscopically, parathyroid adenomas are made up of any one or a combination of all the various cell types normally found in the parathyroid gland (For information concerning the normal histology of the parathyroids the reader is referred to *Gilmours* comprehensive work on the subject (10) These cell types are chief-cells, transitional water-clear cells, water clear cells, and oxyphile cells, most adenomas being composed of chief-cells or a mixture of chief-cells and transitional water-clear cells

According to *Rucart* (15) all the parenchymal cells are developed from a common cell, *cellule fondamentale*, which is a chief cell This cell may develop in two directions, one, *lignee claire*, leads to development of water-clear cells, the other, *lignee sombre*, leads to oxyphile cells over pale oxyphile cells The cells of the *lignee claire* secrete parathyroid hormone, while the cells of the *lignee sombre* secrete a substance which is not parathyroid hormone in the usual sense The *cellules fondamentales* probably secrete both substances

Pure oxyphile and pure water-clear adenomas are not rare There is some difference of opinion as to the hormonal activity of pure oxyphile adenomas *Sommers & Young* (16) report 31 cases of oxyphile adenomas, of which eight had primary hyperparathyroidism However, in Sommers and Young's own two cases the adenomas were not composed of oxyphile cells throughout, but also contained chief cells Thus the hyperparathyroidism in these cases may have been caused by the chief-cells in the adenomas Most authors do not believe that pure oxyphile adenomas may cause hyperparathyroidism (6), (18), (12)

Many authors have found some relationship between the type of parathyroid adenoma and the clinical picture Water-clear cell adenomas are probably associated with a higher serum calcium level than other types (18) Adenomas containing many giant nuclei are stated to be

1
Facts of the 35 Cases

Size	Weight grams	Clinical features	Serum calcium in mg per cent
2 5 × 3 × 3 cm	11 25	halisteresis	15 1 16 1
0 6 × 2 × 2 cm		nefrolithiasis	10 0–14 9
5 × 3 3 × 1 5 cm	12	*ostitis fibrosa* and nephrocalcinosis	13 5 17 3
5 × 3 5 × 1 5 cm	*17*	*ostitis fibrosa*	*12 7 13 7*
4 5 × 2 × 1 cm	*5 2*	*halisteresis* and nephrocalcinosis	16 3 18 6
3 × 2 × 1 cm	5	nephrolithiasis	13 4 15 3
4 × 2 × 1 5 cm	8	halisteresis	13 9–14 9
8 mm in diameter		halisteresis and nephrolithiasis	11 6 13 1
double a normal parathyroid gland	0 38	nephrolithiasis	13 0–14 4
14 × 7 mm		*halisteresis and* nephrolithiasis	11 9 13 5
7 × 4 mm 13 × 4 mm	1 3 1 4	nephrocalcinosis	14 1 15 4
15 × 9 × 8 mm	0 45	halisteresis and nephrocalcinosis	12 7–13 4
3 × 4 × *6 cm*		ostitis fibrosa	18 3–19 6
4 × *3 × 3 cm*		ostitis fibrosa and nephrolithiasis	15 0–18 6
3 × 2 cm		ostitis fibrosa and nephrocalcinosis	14 4–15 6
the size of a hazelnut		halisteresis *and* nephrolithiasis	12 4–14 3
"twice the size of a pea	1 5	nephrolithiasis	11 7–13 5
2 × 3 5 × 6 cm the size of a hazelnut kernel		ostitis fibrosa	14 2–16 1
"3 cm long "3 cm long" 8 × 2 × 0 7 cm	5 5	nephrolithiasis	12 6–13 9

TAB[illegible]
The Most Import[illegible]

Case no	Journal no	Sex	Age	Gland of origin and location
1	157/41	♀	43	li, at the left inferior thyroid pole
2	1230/51	♀	35	ls, at the left superior thyroid pole
3	14/62 63	♂	29	ri behind right thyroid lobe between oesophagus and vertebral column
4	2230/51	♂	51	ls, in the posterior mediastinum on the left side of the tracheal bifurcation
5	57/62–63	♀	33	rs at right thyroid upper pole laterally of oesophagus
6	40/62 63	♂	53	*li just behind the left inferior* thyroid pole
7	2311/52	♀	52	ls behind the left thyroid lobe extending down in the superior mediastinum
8	312/62–63	♀	65	ri behind the right lower thyroid pole
9	1372/53	♀	69	rs at the right upper thyroid pole
10	1997/53	♀	59	li at the left inferior thyroid pole
11	173/62 63	♂	47	rs at the right upper thyroid pole ls at the left upper thyroid pole
12	2066/54	♂	56	ri right side of the superior mediastinum
13	2575/54	♀	43	ri at right inferior thyroid pole mediastinum on the right side of the tracheal bifurcation
14	726/62 63	♀	63	ri, from the posterior border of the right thyroid lobe extending down between oesophagus and trachea
15	97/62 63	♀	58	r in the right thyroid lobe
16	176/62–63	♀	60	rs at the right superior thyroid pole
17	926/59	♀	38	*ri at the right inferior* thyroid pole
18	1705/61 62	♀	57	mediastinum posterior mediastinum medially on the apex of the left lung li at the left inferior thyroid pole
19	230/60–61	♀	53	ri at the right inferior thyroid *pole* li at the left inferior thyroid pole rs posterior mediastinum at the bifurcation of the trachea

cont)

Size	Weight grams	Clinical features	Serum calcium in mg per cent
3 × 5 × 1 cm		nephrolithiasis and an epulis	11 1 11 4
3 × 4 × 4 cm		ostitis fibrosa	13 8–15 1
2 5 × 2 × 1 5 *cm*	8	nephrolithiasis	14 1–15 4
2 5 × 2 × 2 cm	6		
1 5 × 1 5 × 1 cm		ostitis fibrosa and nephrocalcinosis	12 5–15 3
2 × 2 × 4 cm	3	nephrocalcinosis	15 2 17 9
2 × 4 cm		nephrolithiasis	11 6–12 7
the size of a plum"	9	nephrolithiasis	16 6–18 2
"the size of the tip of a little finger"		nephrolithiasis	10 6–12 2
2 cm in diameter		nephrolithiasis	15 4–16 2
the size of a hazelnut"		nephrolithiasis	14 7–15.3
5 × 1 5 × 3 cm		halisteresis and nephrolithiasis	11 6–12 6
"the size of a pigeons egg"	10	nephrolithiasis	10 3 14 4
3 × 2 5 × 2 cm	10	lowered concentration power in the kidneys	14 5–15 5
	5	halisteresis	11 6–13 0
1 5 × 0 5 cm 1 5 × 0 5 cm		nephrocalcinosis	12 4–13 8
4 7 × 2 2 × 1 2 cm	7 4	ostitis fibrosa	13 2 14 8

superi r etc In the same column the location of the adenoma is given The most impor- features" The last column contains the preoperative serum calcium values as the highest

TABLE

Case no	Journal no	Sex	Age	Glan l of origin an l locat on
20	404/62 63	♀	33	ri at the right inferior thyroid pole
21	54/62 63	♂	57	ri behind the right inferior thy roid pole extending down in the superior mediastinum
22	362/62 63	♂	36	l hour glass formed intumescence in the left thyroid lobe r, hour glass formed intumescence in the right thyroid lobe
23	368/58	♀	60	li in the superior mediastinum between the lower left thyroid pole and aortic arch
24	288/62 63	♂	28	li behind left common carotid ar tery in front of the verebral trans verse processes
25	665/62 63	♀	22	li at the lower left thyroid pole
26	1685/61 62	♂	63	li between the lower left thyroid pole the trachea and the carotid sheath
27	623/62 63	♂	37	rs in the right superior thyroid pole
28	6/62 63	♀	35	li behind the middle of the left thyroid lobe on the trachea
29	731/60 61	♀	24	li at the lower left thyroid pole between carotid artery and jugular vein
30	1565/60–61	♀	40	ls behind the left thyroid lobe from the upper pole extending down in the superior mediastinum
31	1258/62 63	♂	49	r in the right thyroid lobe
32	1473/61 62	♂	59	li pulsating tumour in the neck
33	1555/61 62	♀	64	r in the right thyroid lobe
34	756/62 63	♂	45	li at the lower left thyroid pole ri at the lower right thyroid pole
35	1423/62 63	♀	49	li in the superior mediastinum on the left side of the oesophagus over the aortic arch

In the fifth column the gland of origin is given as li rs etc i e left inferior right tant clinical features concerning osseous and renal involvement is found under clinical and lowest values measured

RESULTS

Of the 35 patients 34 had one or more adenomas while only one patient was found to have primary hyperplasia and hypertrophy of the water-clear cell type. No case of carcinoma or chief-cell hyperplasia was found. A total of 43 parathyroid enlargements were removed from the 35 patients. Their distribution is given in Table 2.

TABLE 2
The Distribution of 43 Parathyroid Enlargements in 35 Patients

	No of patients
Single adenomas	29
Double adenomas	4
Triple adenomas	1
Primary water-clear cell hyperplasia and hypertrophy	1*

* In this patient three enlargements were found representing the fused right and left glands.

Adenomas

As will be seen from Table 1, the size of the adenomas vary greatly, the largest measuring 3 × 4 × 6 cm and the smallest having a diameter of only 8 mm. It has been possible to determine the gland of origin of almost every adenoma. In the cases in which the adenoma was a little dislocated, it was often connected with the gland of origin by a thin stalk. When the location of the adenomas is tabulated a certain disproportion appears between the number developed in the superior glands and the number developed in the inferior ones. It will be seen that more than two thirds of the adenomas are situated in the inferior glands or developed from these (Table 3).

TABLE 3
The Gland of Origin of 40 Adenomas in 34 Patients

	Right	Left	Mediastinal	Intraglandular
Superior	6	5		
Inferior	11	14	1	3

As intraglandular adenomas are grouped adenomas embedded in the lateral thyroid lobes. The gland of origin of intraglandular and one of the mediastinal adenomas could not be determined.

Six of the adenomas were found in the mediastinum. Three of these were connected to one of the superior thyroid arteries.

By far most of the adenomas consist of chief cells or transitional water-clear cells or a mixture of these (Figs. 1, 2, 3, 4).

None of the adenomas were made exclusively of one cell type. Below the adenomas are listed according to the dominating cell type or cell

The arrangement of the cells varies Solid arrangement alternates with acinar or cystic pattern *Rogers & Keating* (14) emphasize the characteristic basal orientation of the nuclei producing a pattern resembling branches of berries

Carcinoma

Carcinoma of the parthyroid gland is rare *Black & Ackerman* (1), in a review of alleged parathyroid carcinomas all presenting hyperparathyroidism, reject 19 out of the 26 cases hitherto reported The remaining seven cases all showed metastases or evidence of local invasion at the time of the original operation

The parathyroid carcinoma grows slowly and first metastasizes to the regional lymph nodes One case with metastases to liver and lung has been reported by *Ellis & Barr* (9)

In addition to the types of *hyperparathyroidism described* above, new types have been reported In 1952 *Underdahl et al* reported eight cases of multiple endocrine adenomas in which the parathyroids, pancreatic islets, and pituitary were involved The patients all had primary hyperparathyroidism and hypoglycaemia and various endocrine manifestations of their pituitary adenomas *Cope et al* in 1958 added their description of a new disease entity, namely primary chief-cell hyperplasia, and reported eight cases The primary chief-cell hyperplasia affects all four parathyroid glands and is often associated with tumours of pancreatic islet tissue and pituitary, the patients clinically showing *hyperparathyroidism together with various endocrine abnormalities* This new entity is probably identical with the above mentioned multiple adenomatosis

MATERIAL AND METHODS

The material for the present study has been obtained from the surgical department C at the University hospital in Copenhagen Since 1940 35 patients with hyperparathyroidism have been operated with resultant cure of 33 patients Two patients died (cases no 15 and 23), and the adenomas were found at autopsy For detailed information concerning the clinical and surgical aspects of the present cases the reader is referred to an article by *Dahl Iversen E* (7) and to *Lockwood K & Transbol I* (11)

At the operation all four parathyroid glands have been inspected and palpated Where no parathyroid lesions has thus been discovered the rest of the neck and the superior mediastinum has been further explored from the neck In six cases the patient was subjected to thoracotomy in search of an ectopic adenoma

The removed parathyroid lesions have been measured and in half of the cases weighed at the time of the operation After fixation in neutral formalin the tissue has been embedded in paraffin Sections have been stained with Haematoxylin *Eosin and* with Van Gieson Hansen stain All the paraffin blocks have been available for the present study and several sections from each have been examined

Above in the review of *the literature the criterions I follow in diagnosing the* parathyroid lesion have been outlined In a few cases it has been doubtful whether the lesion represented a primary hyperplasia and hypertrophy of the water clear type or a water clear cell adenoma These cases will be discussed in connection with *primary* hyperplasia and hypertrophy In the list given below (Table 1) the size and the weight (if the adenoma has been weighed) is given for each case



Fig 3

Case 13 f cus of water clear cells in chief cell adenoma The cells are large roun l or p lyg nal w th va uolated clear cytoplasm and a small round nucleus (× 140)

Fig 4

Ca e 14 xyph le cells The cells are p lygot al with clear cut cell l undaries and pal xyphile c t pla m N te the trabecular pattern (× 140)

Fig 1

Case 13 chief cell adenoma The small cells are in trabecular arrangement (× 140)

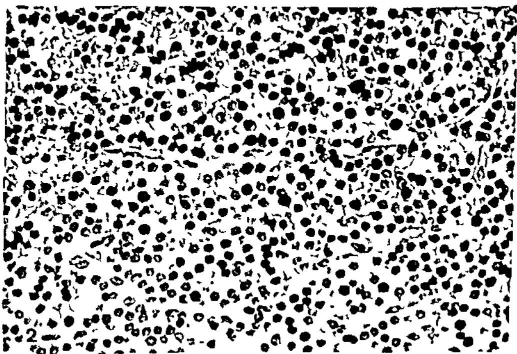


Fig 2

Case 16 from a transitional cell adenoma The cells are small and show a moderately vacuolated cytoplasm (× 350)

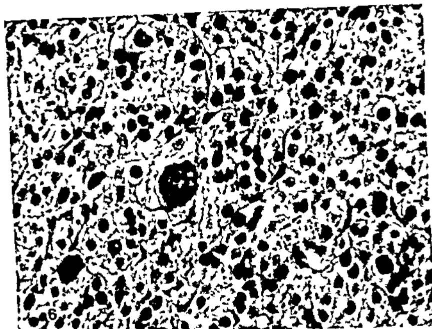


Fig 6

Case 19 giant nuclei found in another part of the adenoma shown in Fig 5 This patient had three adenomas all containing giant nuclei (× 350)

The parenchymal cell nuclei showed slight polymorphism in six of the adenomas and pronounced polymorphism in three (Fig 5), while some degree of nuclear pycnosis has been observed in about half of the adenomas A characteristic nuclear feature is the occurrence of giant nuclei Nuclei with a diameter larger than thrice the normal have been termed giant nuclei and were found in 20 of the adenomas (Fig 6) However, any of the adenomas studied showed at least some degree of variation in nuclear size

These features, nuclear polymorphism and giant nuclei, were found to occur independently in the adenomas, sometimes together, sometimes separately No mitosis was encountered in any of the 40 adenomas studied nor any evidence of capsular invasion, and the clinical course has invariably been benign

The arrangement of the parenchymal cells is generally in solid islands or trabeculae (Figs 2 and 4) In addition to this, six adenomas showed abundant acini, six showed abundant follicles, and in four a cystic pattern was the prominent feature (Figs 7, 8, 9) A delicate vascular stroma separates the trabeculae and islands of cells Generally the stroma is scanty connective tissue rich in capillaries and sinusoids A thin well defined capsule has been found round almost every adenoma A *few of these* showed a thin rim consisting of normal parathyroid tissue outside the capsule (Fig 10)

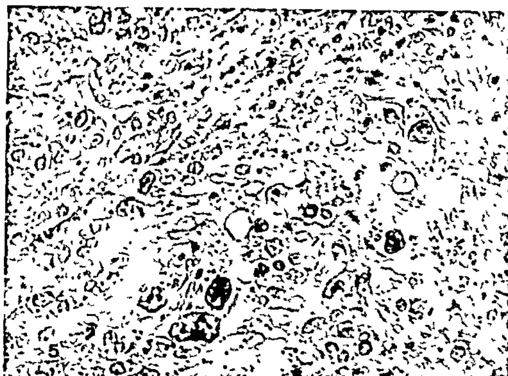


Fig 5

Case 19 severe nuclear polymorphism and giant nuclei In this adenoma several foci of nuclear polymorphism were prevalent No mitosis or evidence of capsular invasion was found (× 350)

types (Table 4) In this table adenomas are only counted as containing two or three types of cells in so far as these occur in a fair proportion in the adenoma, *i e* not less than approximately 20 per cent Where only a small percentage of the cells differ from the others, the adenoma is counted as built up of only one cell type

It will be seen that most of the adenomas are composed chief cells or transitional water-clear cells or a mixture of these, while there is no significant difference between the serum calcium values of the various groups

TABLE 4

The Adenomas are Grouped According to Dominating Cell Types For each Group the Mean Serum Calcium Value is Listed

Cell type or types	No of adenomas	Mean serum calcium (mgpc)	No of patients
Chief cells	16	14 3	16
Transitional water clear	9	14 2	6
Chief + trans water clear	6	14 4	4
Chief + oxyphile	5	14 5	4
Water clear	2	12 8	2
Three cell types	3		3

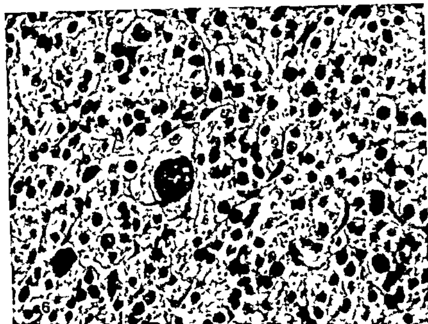


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Fig 7

Case 15 acinar pattern Regular acini some with a small lumen Several foci like this were found in the adenoma (× 140)

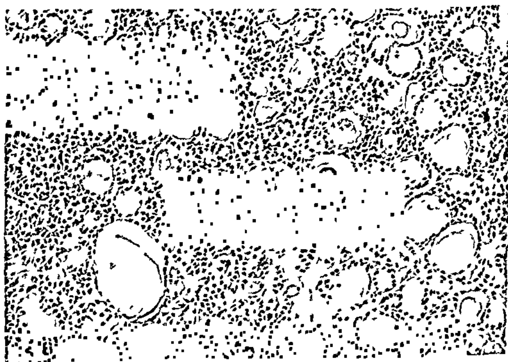


Fig 8

Case 27 focus with follicular structure Follicles of varying sizes filled with colloid like material Such areas may be mistaken for thyroid tissue (× 140)

Fig 9
Case 2 cystic pattern Large cysts filled with granular material The adenoma contained several such foci (× 35)

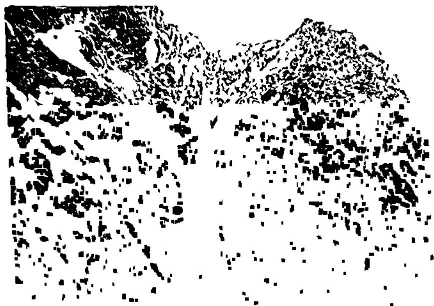


Fig 10
Case 17 The adenoma with its capsule (right) and normal parathyroid tissue containing fat (left) (× 35)

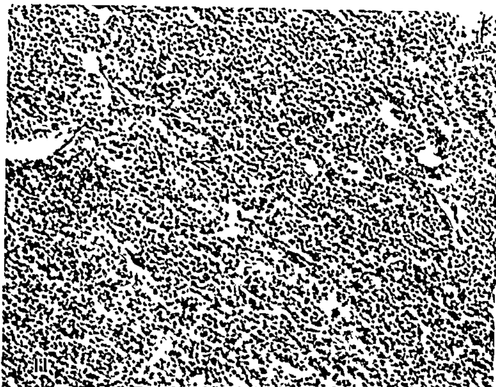


Fig 11
Case 26, parathyroid adenoma built up of chief cells in trabecular arrangement (× 140)

An attempt to find a possible correlation between the weight of the adenoma and the patient's serum calcium value has been made In the 17 cases in which the adenoma was weighed at the time of operation this comparison has been carried out In the diagram below (Fig 16) the weight and serum calcium value found in these 17 patients are given

It will be seen that no correlation is found between weight and serum calcium An equally negative result is obtained when the weight of the adenomas is compared with the corresponding urinary calcium excretion per 24 hours

Case no 26 will be briefly considered here The patient, a 63-year old man, was suffering from renal stones, but showed no sign of bone disease The serum calcium was between 16 6 and 18 2 mg per cent, serum phosphorous 3 8 mg per cent, and the urinary calcium excretion was between 238 and 357 mg per twenty four hours During the exploration of the neck a left inferior parathyroid adenoma was removed together with a firm single nodule in the isthmus of the thyroid gland The thyroid tumour proved to be a papillary thyroid carcinoma, while the parathyroid tumour was a chief-cell adenoma (Figs 11 and 12) However, no lymph node metastases were found, nor any sign of invasion, and the patient was discharged without further treatment No

Fig 12
Case 26 thyroid carcin ma papillary type (× 140)

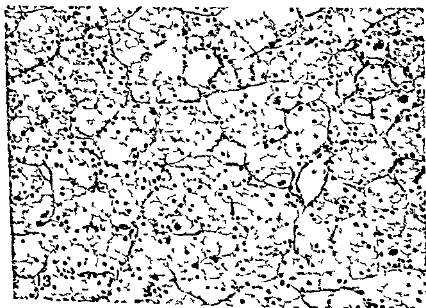


Fig 13
Case 22 Fr m the case of water-clear hyperplasia and hypertrophy All the cells are water-clear the arrangement alveolar (× 140)

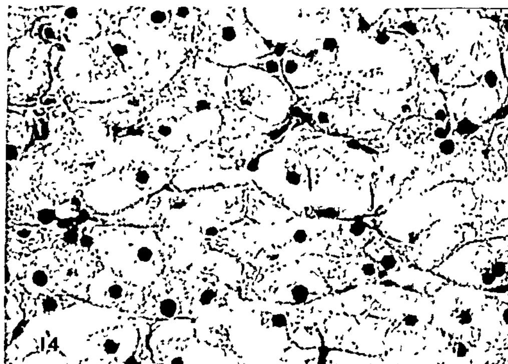


Fig 14

Detail of Fig 13 There is a slight variation in the nuclear size The picture resembles one of hypernephroma (× 350)

Fig 15

From another area of case 22 Note the basal orientation of the nuclei (× 140)

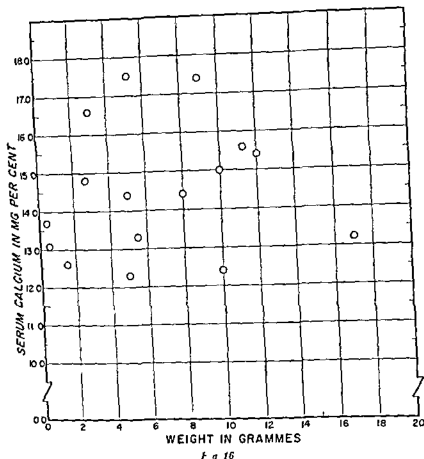


Fig 16

Weight of the adenomas in grammes (abscisse) and the corresponding serum calcium value in mg per cent (ordinate)

sign of recurrence has been detected at the reexamination three years later

Primary Hyperplasia and Hypertrophy

Only one case of primary hyperplasia and hypertrophy of the water clear type was encountered in this series of 35 patients This case (no 22) has caused the surgeons considerable difficulty as the proper diagnosis was not made from the start For long the parathyroid lesion was thought to be two big superior adenomas and it was in fact not until now at the review of the specimens that the diagnosis primary hyperplasia and hypertrophy has been suggested

Case history The patient a 36 year old man was admitted to hospital in august 1937 for renal stones X ray of the kidneys revealed bilateral renal stones but no sign of nefrocalcinosis X ray of the spine and long bone showed no sign of bone

disease The serum calcium level was between 14 1 and 15 4 mg per cent serum phosphorous between 2 4 and 2 5 mg per cent and the urinary calcium excretion per 24 hours was between 191 and 410 mg The diagnosis of primary hyperparathyroidism was made, and at the first operation in august 1957 a rounded tumour measuring 2 5 × 2 × 1 5 cm with a thin downward extension was removed from the upper left thyroid pole This was diagnosed from the frozen section as a parathyroid adenoma built up of water clear cells Postoperatively, however the hyperparathyroidism continued unaltered and the patient was reoperated in april 1958 At this second operation another tumour was removed this time from the lower left thyroid pole The pathological diagnosis of this specimen was adenoma built up of water clear cells Disappointingly, the hyperparathyroidism continued so that a renewed exploration of the neck and the mediastinum was indicated This was carried out in august 1958, when, in the new very fibrosed region an hour glass shaped intumescence was found and removed from the right thyroid lobe the diagnosis being the same as above After this third operation the hyperparathyroidism subsided and the patient is well now four years after the last operation

Looking back, the surgeon concludes that one hour-glass shaped intumescence has been removed in two portions from the left side and a similar one from the right side At none of the operations was any normal parthyroid gland identified Probably the removed parthyroid tissue is the enlarged and fused upper and lower glands on either side

On reviewing the sections it is found that all the parathyroid tissue is composed of large water clear cells throughout (Fig 13, 14 and 15) except for the small bridge connecting the upper and lower left tumours which is built up of water clear cells and smaller cells lying in a fibrous stroma The water clear cells are arranged in an alveolar pattern with interspersed follicles and irregular cysts Basal orientation of the nuclei is a prominent feature The stroma is very scanty and on the surface a thin ill defined capsule is seen There can hardly be any doubt that this represents a case of primary hyperplasia and hypertrophy

Two cases of adenomas having caused differential diagnostic difficulties against primary hyperplasia will be briefly considered here

One case no 31, was found at the operation to have an elongated tumour, the size of a pigeon s egg behind the right thyroid lobe presumably the enlarged and fused right parathyroid glands Microscopically it is built up of large water clear cells in acinar and trabecular pattern although a small focus of chief cells is also seen However, two normal parathyroid glands on the left side were identified by the surgeon Thus primary hyperplasia and hypertrophy was definitely ruled out at the operation table

The other, case no 34 presented two separate tumours each measuring 1 5 × 0 5 cm and located at the lower left and right thyroid poles Histologically the left tumour consisted of water clear cells in an alveolar and cystic pattern together with a few islands of smaller cells The tumour at the lower right thyroid pole was built up of an even mixture of water clear cells transitional water clear cells and chief cells The upper parathyroid glands were not identified with certainty In this case the possibilty of hyperplasia and hypertrophy can be excluded on the basis of the histological picture of the latter of the two described tumours which was largely composed of small cells

SUMMARY AND CONCLUSION

1 The operation specimens from a series of 35 patients suffering from primary hyperparathyroidism has been studied histologically In 29 of the patients a single adenoma was found, four patients had double, and one patient a triple adenoma In one patient the parathyroid lesion was primary hyperplasia and hypertrophy of the water-clear type

2 No case of primary chief cell hyperplasia or multiple endocrine adenomas was found This, however, was not sought for at the time when the patients were in hospital

3 The high frequency of multiple adenomas (14 7 per cent) com

pared with that of other series might be explained by the assumption that some of our multiple adenomas really represent cases of chief-cell hyperplasia

4 More than two thirds of the adenomas were found to have developed in one of the inferior parathyroid glands

5 Histologically most of the adenomas were built up of chief-cells or a mixture of chief cells and transitional water-clear cells No adenoma consisting exclusively of oxyphile cells was found

6 The occurrence of giant nuclei in half of the adenomas and varying degrees of nuclear polymorphism in one fourth of the adenomas in this series tends to emphasize the fact that these features do not denote malignancy

7 No correlation between the weight of the adenomas and the corresponding serum calcium levels has been conspicuous Neither has any connection between the cell type of the adenomas and the serum calcium level been detected

8 One case of parathyroid adenoma and thyroid papillary carcinoma is presented About twenty such cases have been reported in the literature This might warrant the excision of all firm thyroid nodules for histological examination, when found in patients having hyperparathyroidism

9 The single case of primary hyperplasia and hypertrophy is presented The differential diagnosis against water-clear cell adenoma is discussed The possibility of water clear hyperplasia and hypertrophy can be excluded, when normal parathyroid glands are identified at operation or when the histological examination shows the parathyroid tissue to be largely composed of small cells

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ATHEROSCLEROSIS IN AN AUTOPSY SERIES

2 *The Lipoid and Calcium Contents of the Aorta in Childhood*

By

J Chr Geertsen

Received 25 x 63

Virchow (38) considered the fatty streak ("die einfache Fettmetamorphose") that may be found in the aortic intima of juveniles, to be unrelated to the atheromatous process It might develop into a superficial ulceration ("die fettige Usur'), but not into a true atheroma

Although some authors still maintained Virchow's view (f i *Hueck* (18), *Klotz & Manning* (20), *Ribbert* (29), *Sanders* (32)), the opinion gradually gained ground from the beginning of this century that the fatty streak represents an early atherosclerotic lesion (*Anitschkow* (2), *Aschoff* (3), *Lubarsch* (22), *Saltykow* (31), *Schmidtmann* (33), *Zinserling* (40)) This view is widely accepted today (Study Group on Classification of Atherosclerotic lesions (8), *Duff & McMillan* (9))

Fatty streaks may be found very frequently in the aorta of children, even in the first year of life, especially after Sudan staining of the artery (*Kube & Ssolowjew* (21), *Saltykow* (31), *Schmidtmann* (33), *Zinzerling* (40)) On the basis of the enormous material (almost 50 000 aortas from 20 countries) collected for the Deuxième Conference Internationale de Pathologie Géographique held in Utrecht in 1934, *Anitschkow* (2) concluded that the fatty streak is a general occurrence in the aorta of all individuals from the age of 10 years, despite the widely varying dietary and other living conditions in the different countries

The high incidence of fatty streaks in the juvenile aorta has been confirmed by a considerable number of later reports (*Albert* (1), *Galindo Arean, Strong & Baldizon* (10), *Holman, McGill Jr, Strong & Geer* (16, 17), *Mathur, Patney & Kumar* (23), *Murthy, Datta & Ramalingaswami* (24), *Restrepo & McGill Jr* (28), *Robertson* (30), *Strong, McGill Jr, Tejada & Holman* (34), *Strong, Wainwright & McGill Jr* (35) *Tejada & Gore* (36), *Tejada, Gore, Strong & McGill Jr* (37)) The findings in 1,100 aortas from 7 different countries form the basis of *Holman McGill Jr, Strong & Geer's* (16, 17) concept of "The natural history of atherosclerosis" According to this concept, the fatty streak is considered to be the primary lesion in atherosclerosis, formed in the aorta in the first two decades of life

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TABLE 2

The Contents of Lipoid and Calcium in the Aorta of Children Mean and Standard Error of the Mean (Italics)

Age group	No of cases	Tl	Ch	Ph	Ca	ChTl	PhTl	ChPh
12 7 weeks premature	12	11 47 *0 87*	1 15 *0 08*	3 09 *0 25*	0 12 *0 03*	10 2 *0 6*	28 1 *2 6*	0 38 *0 03*
6 weeks and less premature	14	10 48 *0 76*	0 94 *0 07*	2 95 *0 20*	0 10 *0 01*	9 7 *1 1*	29 2 *1 7*	0 33 *0 03*
Mature Stillborn	11	8 *0 44*	0 66 *0 07*	2 33 *0 09*	0 08 *0 01*	8 4 *1 –*	30 – *2 3*	0 30 *0 04*
0 6 days	14	0 86 *0 83*	0 78 *0 05*	2 26 *0 12*	0 14 *0 03*	11 9 *1 –*	34 5 *2 3*	0 35 *0 01*
1 25 weeks	12	5 68 *0 35*	0 52 *0 04*	1 79 *0 10*	0 11 *0 02*	9 3 *0 8*	31 9 *1 –*	0 30 *0 02*
26 51 weeks	6	3 87 *0 37*	0 48 *0 06*	1 25 *0 12*	0 07 *0 01*	12 6 *1 2*	33 3 *2 7*	0 38 *0 02*
1– 4 years	11	3 44 *0 11*	0 36 *0 02*	1 11 *0 05*	0 09 *0 01*	10 7 *0 6*	32 7 *1 7*	0 33 *0 01*
5 9 years	2	4 70	0 63	1 61	0 08	13 4	34 4	0 39
10 14 years	4	5 84	0 88	1 80	0 09	14 9	31 2	0 4[illegible]

Tl — total lipoid, Ch — total cholesterol, Ph — phospholipoid, Ca — calcium: percentage of the aortic dry weight

ChTl, PhTl: total cholesterol and phospholipoid as a percentage of total lipoid

ChPh — cholesterol/phospholipoid ratio

In this connection it is immaterial that the opinions are not unanimous as to which arterial change is the primary lesion in atherosclerosis. The point is that if the fatty streak is accepted as an atherosclerotic lesion at all, then is must be accepted that atherosclerosis starts in early childhood, a fact that prompted *Holman* (14) to ask whether atherosclerosis is a paediatric nutritional problem

In view of this, and in view of the great attention shown to the chemistry of atherosclerosis, it is remarkable that chemical analysis of the arteries from children should have been carried out only occasionally (*Buck* (5), *Buck & Rossiter* (6), *Bottcher & Van Gent* (7), *Hevelke* (13)), and that no series of systematic analyses can be found in the literature.

MATERIAL

The aortas from 86 children (50 males and 36 females), ranging in age from birth to 14 years, have been analyzed The causes of death are presented in Table 1, and the age distribution appears from Table 2 Twenty-six children were prematures, and these have been grouped according to the number of weeks of prematurity, namely one group born 12–7 weeks before term (mean 8 weeks), and one group born 6 weeks or less before term (mean almost 5 weeks) Five of these children were stillborn, and 21 had been alive for from a few minutes to 6 days No distinction has been made between premature and mature children after the first week of age

TABLE 1
Causes of Death

Disease group	*No of cases*
Acute hepatitis	1
Malignant tumour	3
Benign tumour	1
Meningitis	1
Pneumonia	8
Bronchitis	2
Appendicitis	1
Strangulation ileus	1
Complications in gestation, at birth and in puerperium	16
Congenital malformations	19
Birth trauma, asphyxia and pulmonary atelectasis	30
Sudden natural death	1
Sudden violent death	2

[illegible] ...lysed Total lipoid (Tl) total cholesterol [illegible] ..., and the contents have been expressed [illegible] ...rta From the results of these analyses the following values have been calculated. Total cholesterol and phospholipoid as a percentage of total lipoid (ChTl and PhTl), and the cholesterol/phospholipoid ratio (C[illegible] been previously described (*Giertsen* (11)) [illegible] aortas were macroscopically normal, except 3 of [illegible] 14 years which displayed small fatty streaks Sudan-staining was not employed as this procedure removes some of the lipoid (*Holman, McGill Jr, Strong & Geer* (15))

the quality is kept remarkably constant as the ChTI, PhTI, and ChPh values only vary within rather narrow limits However, at the same time as the quantity starts to increase, and as a consequence of the disproportionally rapid increase in the Ch value the quality of the lipoid changes Thus the ChTI value increases more than the PhTI value and a distinct increase of the ChPh value is noted The number of cases is admittedly small in the two last age groups but the trend of the quantitative and qualitative changes seems clear

The chemical findings in the aorta of identical twins (Table 3) Both sets of twins were born at term In the first set the twins died within 12 hours of each other one from an intracranial haemorrhage the other from pulmonary atelectasis The findings in these two are practically identical The TI values are lower and the ChTI and PhTI values higher than the means for the age group concerned but the ChPh values closely correspond to the mean

In the second set both died from endocardial fibro elastosis The findings in the youngest twin correspond well with the means for the age group In the oldest the TI and Ph values are slightly lower, and the Ch value markedly lower than the means Concequently the ChPh value is low

The chemical findings in the aorta of a mother and her foetus (Table 3) The mother was a 38 year old primipara who developed an intoxication with eclampsia and died from an intra pontine haemorrhage 4 weeks before term without having been delivered Her aorta displayed a few fatty streaks The quantitative values in these two aortas are hardly commensurable because of the great difference in size of the artery (see discussion) It is evident however that the quality of the lipoid in the mother s aorta has nothing in common with that of the foetus aorta Thus the mother s ChPh value is more than 3 times as high as that of the foetus

DISCUSSION

The interpretation of the decreasing quantity of the lipoid in the aorta during the first years of life is not unequivocal In the period concerned the aorta grows considerably in size Thus the dry weight of the artery increased 6 times from a mean of 0 13 g in the mature stillborn child to a mean of 0 80 g in the 3–4 year age group It seems reasonable that the main part of the increase in weight is due to the growth of the media If the lipoid is equally distributed throughout the entire aortic wall (i e intima + media) then the decrease is real On the other hand if the child s intima contains more lipoid than the media as the normal looking adult intima does (*Weinhouse & Hirsch* (33)) then the inclusion of the media in the calculations will tend to lower the mean lipoid content of the artery This has previously been called the masking effect of the media on the percentage figures for

RESULTS

The results are presented in Table 2 Only the first, second, and fourth age-groups comprised males and females in such proportions that the individual results obtained in the two sex groups could be compared (6-6, 8-6, and 7-7 males and females, respectively) No statistically significant sex difference could be found Therefore, the sexes have been combined in the presentation of the results The separate findings in two sets of identical boy twins, and in a mother and her foteus are presented in Table 3

TABLE 3
The Contents of Lipoid and Calcium in the Aorta of Two Sets of Identical Boy Twins and in a Mother and her Foetus

	Age	Tl	Ch	Ph	Ca	ChTl	PhTl	ChPh
First set	57 hrs	5 08	0 71	2 18	0 09	14 –	42 9	0 33
of twins	69 hrs	5 21	0 76	2 50	0 07	14 6	48 –	0 30
Second set	38 hrs	7 56	0 92	2 78	0 04	12 2	36 8	0 33
of twins	26 days	6 41	0 38	2 05	0 05	5 9	32	0 19
Mother	38 yrs	8 71	2 20	2 64	0 56	25 3	30 3	0 86
Foetus	4 wks premature	11 64	0 68	2 57	0 03	5 8	22 1	0 26

Tl = total lipoid, Ch = total cholesterol, Ph = phospholipoid, Ca = calcium — percentage of the aortic dry weight
ChTl, PhTl — total cholesterol and phospholipoid as a percentage of total lipoid
ChPh = cholesterol/phospholipoid ratio

The quantity of the lipoids It appears that the "youngest" children, that is those 12 7 weeks premature, have the highest lipoid content From then on the Tl-, Ch and Ph-values decrease distinctly, although slightly with *increasing age, reaching a minimum in the age-group* 1–4 years The Ch-value admittedly increases slightly in the mature child during the first week, but this does not affect the general trend of the figures The total decrease in the lipoid content is substantial, as the 1 to 4 year-old child's aorta contains only about one half of the lipoid amount in the newborn, mature child, and only about one third of that found in the child borne 12–7 weeks prematurely

From the minimum values in the 1 4-year group the lipoid values again increase with age However, the Ch-value increases more rapidly than the Tl- and Ph-values, and in the age group 10 14 years it has already passed the figure relating to the mature, stillborn child, whereas both the Tl- and Ph-values lag behind

Calcium It appears that the calcium content is more or less constant in the entire age-period examined

The quality of the lipoid In contrast to the marked and consistent changes in the quantity of the lipoid with age in the first five years,

and shows a marked decrease in the first year From then on it is more or less constant Consequently the serum ChPh value increases markedly in the first year and then it remains constant up to the age of 14 years although it does not reach Barr s *normal adult value Thus, it* seems that the infant loses its serum lipoid characteristics already in the first year None of the serum values equalize those found in the aorta It may be however that separate analyses of the intima and media might show *values differing from those observed* in the present investigation

TABLE 4

The Interrelationship between Total Lipoid Total Cholesterol and Phospholipoid in Serum from Infants and Children Mean Values Calculated from Rafstedt s (27) Tables

Age	No of cases	ChTl	PhTl	ChPh
Newborn	32	24 7	43 6	0 57
0– 6 days	43	23.3	41 6	0 57
1 25 weeks	41	21 7	32 7	0 68
26 51 weeks	46	21 8	30.3	0 73
1 4 years	6	24 1	30 2	0 81
5– 9 years	13	22 6	28 1	0 81
12 14 years	*6*	*22 7*	*29 1*	*0 78*

ChTl and PhTl = total cholesterol and phospholipoid as a percentage of total lipoid
ChPh = cholesterol/phospholipoid ratio

It is not justifiable to draw any inferences with regard to the significance of the changes in the lipoid composition in the serum and the aorta in the pathogenesis of atherosclerosis and with our present knowledge it seems that any conclusion would be hasty It may even be that the changes have no bearing on atherosclerosis at all as they may only reflect an adaptation process of the child to the extra uterine life

On the other hand fatty streaks are found in the aorta in every individual over 3 years of age (*Holman McGill Jr, Strong & Geer* (16 17)) *the aortic ChPh value starts to* increase in the second half of the first decade this increase continues almost linearly with increasing age in the adult (*Giertsen* (12)) and the ChPh value is a valid index for the amount of atherosclerosis present in the arteries (*Giertsen* (11)) From these facts it does not seem *unreasonable to put forward* the hypothesis that the changes observed in the lipoid composition of the aorta are an expression of the starting point of atherosclerosis namely at the age of about 5 years It should be emphasized however that due to the small number of cases in the critical age groups in this investigation the validity of this hypothesis will have to be tested by further investigations It should also be emphasized that the changes observed are not necessarily the esssential ones They may only indicate the effect of the causal factors

the lipoid (*Giertsen* (11)) It may even be possible that the lipoid content in the intima is constant in the age-period concerned

We do not know the relative proportions of the lipoid in the intima and media of children If they differ appreciably, the values expressing the quality of the lipoid will also be influenced by the growth of the artery Here we can only establish the fact that the quality of the lipoid remains unchanged in the combined intima-media in the first few years of life

In the normal adult aorta the calcium is mainly present in the media (*Weinhouse & Hirsch* (39)) If this applies to the child's aorta as well, the percentage calcium content will not be appreciably influenced by the growth of the artery Significantly enough the calcium content is practically constant in the child's aorta

In any case, normal values for the quantity of the lipoid in the child's aorta cannot be established without taking the age of the child into consideration, even in the first few years of life In so far as the aorta is considered normal during these years, the normal quality can be established, namely as the mean of the values found in the 54 aortas contained in the "mature, stillborn" group to the 1–4 year age group These means are ChTl 10 5, PhTl 32 5, and ChPh 0 33 The corresponding normal calcium content is 0 10 per cent of the dry weight of the aorta

Despite a possible masking effect on the percentage figures due to the continued growth of the aorta, a definite increase in quantity and a change in quality occur in the second half of the first decade Something obviously happens to the lipoid in the aorta at the age of about 5 years Bearing in mind the filtration theory of the pathogenesis of atherosclerosis so strongly advocated by many authors (see f i *Page* (25, 26)), it may be opportune to consider the lipoid composition of the blood in the age-period concerned *Barr* (4) called attention to the striking similarity between the lipoid composition of the blood in the newborn and that in the rabbit and the rat, which appear to be immune to spontaneous atherosclerosis He reported, among other things, a serum ChPh-value of 0 56 in the newborn, 0 58 in the rat, and 0 67 in the rabbit, against a value of 0 90 in the young adult human Barr stated "It would seem then, from the standpoint of analyzable chemical factors, that the newborn baby closely simulates or resembles the rabbit and rat, that sometime during childhood, and perhaps quite early, these characteristics are lost"

Thanks to *Rafstedt's* (27) comprehensive studies, data are available on the lipoid composition of the serum in infants and children The serum ChTl-, PhTl-, and ChPh-values have been calculated by the present author from Rafstedt's tables, and the results are presented in Table 4 It appears that the serum ChTl-value is more or less constant in the first 14 years of life, about twice as high as the aortic value The serum PhTl-value is about one half as high as the aortic value at birth,

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It seems justifiable to suggest that a large part of future athero sclerosis research should be concentrated on the conditions in child hood

SUMMARY

The contents of total lipoid, total cholesterol, phospholipoid, and calcium have been determined in the aorta from 86 children ranging in age from birth to 14 years The following values have been calculated from the results of the analyses: Total cholesterol and phospholipoid as a percentage of total lipoid, and the cholesterol/phospholipoid ratio

The quantity of the total lipoid as well as of its two fractions de creases steadily in the first years of life The percentage values, how ever, may be influenced by the growth of the aorta In the same age period the quality of the lipoid remains unchanged The calcium con tent is constant in the entire age-period examined

In the second half of the first decade the contents of total lipoid and its fractions increase, but cholesterol increases more rapidly than total lipoid and phospholipoid The result is a change in the quality of the lipoid, expressed by a rise in the cholesterol/phospholipoid ratio

Two sets of identical twins were examined In the first set the twins died within 12 hours of each other, and the chemical findings in this set were almost identical The other two died within 3½ weeks of each other In the oldest twin the cholesterol content and the cholesterol phospholipoid ratio were low The findings in a mother and her foetus differed considerably

It is recognized that normal values for the quantity of the lipoid in the aorta cannot be established without taking the age of the child into consideration Normal values for the quality of the lipoid and for the calcium content are given

The lipoid composition in the aorta from children was compared with that found in the serum by another author A rise in the chole sterol/phospholipoid ratio was found in both, but the ratio begins to increase a few years earlier in the serum than in the aorta, namely al ready in the first year No definite conclusions can be drawn from this comparison

It is suggested that the changes observed in the lipoid composition of the aorta may indicate the starting point of atherosclerosis, namely at the age of about 5 years Further, a large part of future athero sclerosis research ought to be concentrated on the conditions in child hood

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THE DISTRIBUTION OF LACTATE AND NADH TETRAZOLIUM REDUCTASE[1] ACTIVITY IN THE FOETAL HUMAN GASTRIC MUCOSA

By

MIKKO NIEMI and PENTTI SALENIUS

Received 13 xi 63

The gastric mucosa of both adult animals (*Ruthenburg et al* 1953) and man (*Niemi et al* 1960, *Planteydt & Willighagen* 1960) has been shown to contain many of the histochemically demonstrable tetrazolium reductase activities Most of these oxidative enzymes are particularly active in the parietal cells of the gastric gland Moreover, correlations have been observed between the acid production of the gastric mucosa and its content of parietal cells (*Myren & Semb* 1962) and their activity to reduce succinate (*Villarreal & Burgos* 1955)

The results of the enzymatic histochemical staining of embryonic gastric mucosa differ, however, considerably Succinic dehydrogenase activity has been reported to appear in the gastric mucosa of the rat only postnatally (*Vollrath* 1959) On the other hand, the gastric mucosa of human foetuses has been found to contain this enzymatic activity already at the 9th or 10th intrauterine week, but while some have observed no special localization of the enzyme (*Rossi et al* 1954, 1957), *Salenius* (1962) has shown it to be concentrated at the bottom of the embryonic gastric pits

The study to be reported here is an extension of the latter study, and

[1] In current histochemical practice oxidative enzymic activity is usually demonstrated with the aid of various tetrazolium salts The enzymes responsible for the reduction of tetrazoles to formazans are named according to the substrate used although it is not clear at what stage of the [illegible] razoles are [illegible] 'etrazolium [illegible] ig enzymes [illegible] instances [illegible] of histochemically demonstrated [illegible] dehydrogenases are compared with the biochemically estimated dehydrogenase activity and it seems therefore to be of importance to accept the terminology proposed

Histochemists have also often used the designation "diphosphopyridine nucleotide" (DPN) although the Commission on Enzymes of the International Union of Biochemistry has suggested the name "nicotinamide adenine dinucleotide" The regeneration of reduced pyridine nucleotide has likewise been ascribed to a "DPN diaphorase" although the term diaphorase has been appropriated for another enzyme by the biochemists

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The results of the enzymatic histochemical staining of embryonic gastric mucosa differ, however, considerably. Succinic dehydrogenase activity has been reported to appear in the gastric mucosa of the rat only postnatally (*Vollrath* 1959). On the other hand, the gastric mucosa of human foetuses has been found to contain this enzymatic activity already at the 9th or 10th intrauterine week, but while some have observed no special localization of the enzyme (*Rossi et al* 1954, 1957), *Salenius* (1962) has shown it to be concentrated at the bottom of the embryonic gastric pits.

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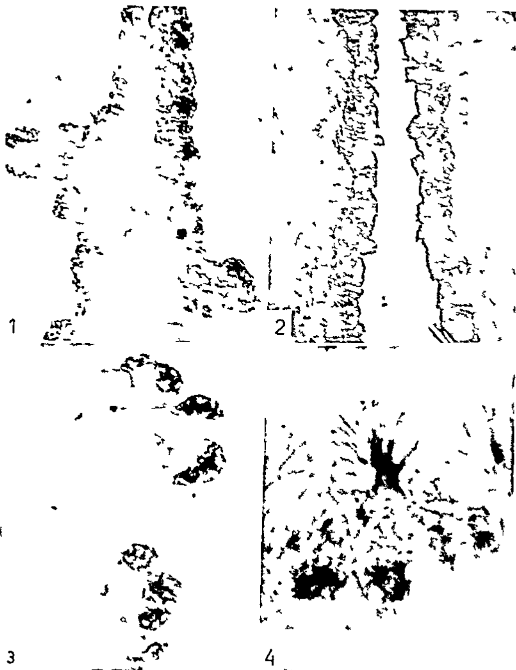
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Figs 1 4

Fig 1 NADH tetrazolium reductase (DPN diaphorase) activity in the gastric mucosa of a 70 mm long human foetus (about 9 weeks of age) Note the clear activity maximum at the bottom of the developing gastric glands MTT cobolt method × 120

Fig 2 Stomach mucosa of the same foetus as in the previous picture but tetrazolium reductase activity demonstrated now using lactate as a substrate (lactate dehydrogenase) Formazan is evenly deposited throughout the epithelium but the luminal border of the epithelium seems to be slightly more heavily stained than the rest of the mucosa Nitro Bt method × 120

Fig 3 Gastric mucosa of a human foetus of CH 140 mm (about 13 weeks Most of the NADH reductase activity is localized in the developing parietal cells in the bottom of the gastric pits MTT cobolt technique × 250

Fig 4 Lactate dehydrogenase activity in the gastric glands of the same foetus as in Fig 3 The activity shows even here a tendency to concentrate at the bottom of the crypts × 450

it was our purpose to observe whether other oxidative enzymes are present so early in the human foetal stomach and whether they may indicate also an early differentiation in the primitive gastric pits

MATERIAL AND METHODS

The material cons sts of 20 spec mens from 7 human foetuses The age of the foetus is g en n weeks of gestat on when calculating the age e ther the menstrua t on age or the length of the foetus (crown heel) v as used From the menstruation age however two weeks were al ays subtracted As a reference sample specimens from an adult human stomach were stud ed s multaneously

Tissue pie es were obta ned in connection with operat ons or premature deliveries a

RESULTS

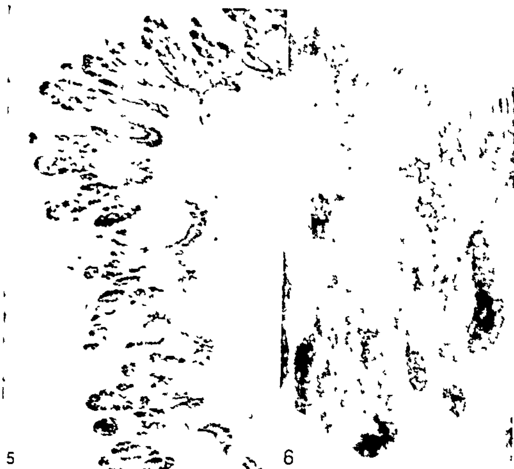
Reducing activity towards both of the substrates used was seen al ready in the youngest foetus of the present series which had a CH length of 70 mm and was 9 weeks of age Lactate dehydrogenase activity was diffusely located in the foetal mucosa (Fig 2) but the NADH de hydrogenating activity could be seen to concentrate already at the bottom of the developing gastric pits Fig 1) although slight activity was also present elsewhere in the mucous membrane

The gastric mucosa of a foetus one week older (CH 95 mm age 10 weeks) showed very similar dehydrogenase activity lactate tetrazolium reductase activity still being quite homogeneously distributed in the epithelium but NADH reductase activity showing a preference for the bottom of the pits

The next two foetuses (CH 140 mm and 145 mm) representing a foetal age of 13 weeks showed different lactate dehydrogenase localiza t on in their gastric mucosa It now demonstrated a tendency for con centration at the bottom of mucosal folds whereas the activity of the cells remaining in the surface epithelium was much less than during the previous stages (Fig 4) This difference was further pronounced in the n xt older foetuses Both in the 16 week (CH 200 mm) and in the 21 week (CH ?80 mm) foetuses the l tat ? ?
cl arly localized in cells v
f parietal cells Th s wa
pared with that seen in a ? ?ult gastric mucosa

The re luctase activity towards reduced NAD had throughout the observation period mainly the same localization as that seen in the youngest of the foetuses However it became quite clear at the foetal age of 13 weeks that this enzymic activity was most pronounced in the d vel ping parietal cells while the surface epithelium was definitely

¹ 3 (4 5 D methylthiaz l 1 2) 2 5 d phenyltetrazolium b r m de



Figs 5 and 6

280 mm long foetus (about 21 weeks of age) NADH dehydrogenase activity demonstrated with MTT cobolt technique An activity maximum in the parietal cells is clearly visible Surface epithelium shows some perinuclear activity Fig 5 × 250 Fig 6 × 400

less active (Fig 3) In older foetuses the NAD reductase activity was quite equal to that of adult stomach mucosa (Figs 5 and 6)

DISCUSSION

Adult human gastric mucosa has been investigated histochemically recently with reasonable thoroughness (*Niemi et al* 1960, *Dawson & Pryse-Davies* 1963, *Correira et al* 1963) From these studies it has become evident that the acid-secreting cells of the gastric glands show intense activity of various oxidative enzymes including lactate and NADH dehydrogenases The rest of the epithelium on the other hand, either shows no activity at all or a moderate activity concentrated in the perinuclear area in contrast to the heavy staining of all the cytoplasm in the parietal cells Moreover, histamine induced acid secretion has been shown to increase the activity of succinate dehydrogenase in

animals (*Villarreal & Burgos* 1955 *Vitale et al* 1956) and both succinate and NADH dehydrogenases in man (*Correira et al* 1963)

Foetal gastric mucosa has been studied histochemically much less The morphological studies of *Salenius* (1962) and others (*Plenk* 1931) have shown that the first signs of cellular differentiation in the embryonic human stomach can be seen at about the age of three months Most observers have agreed that the first differentiated cell type is the parietal cell It is of interest that *Keene & Hewer* (1929) could demonstrate hydrochloric acid in the stomach of an embryo soon after the time when the morphologic differentiation of the gastric parietal cells had been observed

Histochemical studies by *Vollrath* (1959) and *Rossi et al* (1954 1957) have demonstrated succinate dehydrogenase activity to be present in the gastric mucosa during the foetal life of the rat and man In the rat enzymatic activity was very weak before the birth however In the human embryo the activity was present after 11th foetal week but without any specific localization In an earlier communication *Salenius* (1962) was able however to show that in the human embryo of 9 weeks succinate dehydrogenase activity was already present and was most intense in the cells at the bottom of the glandular pits where also the first differentiated parietal cells were found

It is clear from the results presented above that the human foetal stomach already demonstrates signs of epithelial cell differentiation histochemically at the time when the cells of the gastric mucosa are not observed to be differentiated morphologically In the youngest foetus of the present series the NADH dehydrogenase activity was found to have a definite tendency to localize in the primitive mucosal pits where the morphological well differentiated parietal cells were found some weeks later

There were some differences between the localization of tetrazolium reductase activity when lactate or reduced pyridine nucleotide was used as a substrate Dehydrogenase activity towards NADH showed earlier an increased activity in the developing parietal cells whereas lactate dehydrogenase activity remained homogeneously distributed among the epithelium until a few weeks later In this respect both succinate and NADH dehydrogenases behave identically It might be that their differentiation precedes that of the pyridine nucleotide linked oxidative enzymes during embryonic development

SUMMARY

The distribution of tetrazolium reductase activity in the foetal human gastric mucosa was studied histochemically using lactate and reduced pyridine nucleotide (NAD) as substrates (enzymes responsible for staining have also been called lactate dehydrogenase and DPN diaphorase) The embryos studied were of seven different foetal ages the

youngest being 9 weeks old and the oldest 21 weeks Adult human stomach mucosa was used as a reference

Both the oxidative enzymes were already present in the gastric mucosa of the youngest embryo, but while NADH reducing activity was most intense at the bottom of the glandular pits, probably in the developing parietal cells, the lactate-NAD-tetrazolium reductase activity was homogeneously distributed in the epithelium until the foetal age of 13 weeks, whereafter it also was localized mainly in the parietal cells The surface epithelium was seen to lose most of its enzymic activity after the 14th intrauterine week

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PROBLEMS CONCERNING THE DEFINITION AND GRADING OF THE AORTIC ATHEROSCLEROSIS

By

Sv Bertelsen

Received 15 xi 63

The studies of as well the human as the experimental atherosclerosis have increased the interest and the demand for a uniform definition and an exact grading of the atherosclerotic vascular changes

Diverse views regarding this problems have lead many investigators to widely different results The former pathological anatomical definition of atherosclerosis as an intimal, spotted lipoidal degeneration with a secondary thickening and sclerosing of the superficial intima, originates directly from *Virchow*'s (1856) conception and description of the pathogenesis of the atherosclerosis namely, that the fat, secondary to an intimal degeneration, is the essential factor in the development of atherosclerosis and the cause of both the fibrosis and calcareous deposits in the tunica intima

This conception of the nature of the atherosclerosis has caused many investigators of both experimental and human pathology to describe the intimal accumulation of lipid as atherosclerosis, without having ascertained that actual intimal sclerotic changes have occurred

Intensive studies of atherosclerosis during recent years, and particularly the investigations carried out during the last 10-15 years on the ground substance of the aortic wall have induced investigators [illegible]

[illegible]

[illegible] of changes of the intima of arteries (as distinguished from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes" This definition does not touch on the pathogenesis of the atherosclerosis and says nothing of a possible relation between the accumulation of lipids

and carbohydrates, and the fibrosis in the vascular wall The study group seems to include medial changes in the concept of atherosclerosis which is inconsistent with the examination of the present author

When estimating the atherosclerotic degree of a vessel *the biochemical minded investigator* endeavours to grade the changes by means of quantitative and qualitative chemical analyses, while *the pathologist* classifies the atherosclerotic changes into 3 or 4 differences of degree in accordance with the extension of the macroscopic and or microscopic changes *The clinician* on the other hand is confined to evaluate the atherosclerosis of the vessels by means of the subjective and objective symptoms of the patient In spite of the fact that several investigators during the recent decades indirectly have proved that the until now employed pathological-anatomical and biochemical principles hardly are appropriate in a scientific evaluation of the atherosclerotic process these methods have nevertheless been employed numerous times both in the human pathology and in experimental studies of atherosclerosis Most likely this is due to the fact that until now no better grading methods have been available, and consequently large parts of the literature are difficult to estimate, and a comparison between the different results is often impracticable

In order to evaluate the atherosclerotic changes it is imperative to be familiar with the morphologic changes which occur in the vascular wall partly *with increasing age and partly with the development of* athero sclerosis The age changes in the aortic wall denote changes of a uniform nature and appearance which occur both in normal and in atherosclerotic parts of the vessels, while the atherosclerotic changes are specific for the atherosclerotic parts of the vessels

By means of histochemical and chemical methods the present author has examined both the age changes and atherosclerotic changes in the aortic wall The studies are reported in previous papers (*Bertelsen* 1961 a, b c, 1962), and a brief description is rendered

HISTOCHEMICAL EXAMINATIONS[1]

The investigations were made on 163 adult aortas in the age groups 0 90 years furthermore 27 foetal aortas were examined The tissue specimens were embedded in paraffin The acid mucopolysaccharides were stained with toluidine blue alcian [illegible] and Hale s [illegible]lloidal iron stain The neutral mucopolysaccharides were stained [illegible] d both forms of mucopolysaccharides were stained [illegible] and PAS Hale s stain The elastic and collagen [illegible] elastin staining and van Gieson Hansen s staining Lipid was demonstrated with Sudan III and calcium with alizarin red S staining

In newborn infants the elastic fibres in the *tunica media* are closely packed with scanty ground substance among the fibres Already from birth, however, the amount of ground substance is constantly increasing with age, the elastic fibres being gradually split apart Each single

1 Cf the pictures in the references *Bertelsen* (1961a b)

elastic fibre is gradually straightened out and at the same time fragmentated and their course becomes irregular often duplicated with numerous communicants visible between the individual fibres

Simultaneously a vigorous proliferation of cells occurs in the ground substance predominantly of fibroblasts but along the elastic fibres a few muscle cells are visible The collagen fragments observable in van Gieson Hansen s staining are increasing with age in length as well as in number The amount of argyrophile fibres located chiefly along the elastic fibres like a mantle is increasing with age

Histochemical stainings show an increasing amount of acid as well as neutral mucopolysaccharides in the ground substance in the media Staining with Alizarin red S for calcareous deposits shows a considerable accumulation of calcium salts in the media after the second decade At first the calcium salts will appear as granules deposited in the metachromatic ground substance and with increasing depositing the calcium will occupy a steadily increasing part of the interfibrillar space The mineralization appears to start rather diffusely in the intimal half of the media spreading with age throughout the media Sudan III staining shows that the content of lipid in the media is scanty especially in relation to the accumulation in the intima Lipid deposits in the ground substance are observed only in the luminal third of the media and than only when large amounts are present in the intima

Immediately after birth a thickening of the *tunica intima* starts increasing constantly with age and consisting of ground substance and fibroblasts During the intimal proliferation the internal elastic membrane shows a vigorous rupture and fragments of elastic fibrils are visible in the profound part of the intima Moreover an al n lant

a stainings show that during the first and second decades the intimal ground substance chiefly contains acid mucopolysaccharides but after this age also neutral mucopolysaccharides will appear The thickened intima will soon be subjected to a lipid accumulation Starting in infancy it will grow steadily with increasing age The lipid may be intercellular in the so called foam cells , but with increasing amounts it will appear in large extracellular lumps with "foam cells peripherally located Normal intimal tissue shows no sign of reaction in the connective tissue round the lipid accumulation It appears from Alizarin red S staining that neither aortae with normal gross appearance nor normal sections of atherosclerotic vessels show the least trace of calcium deposits in the intima

Comparisons made between sections from aortae with normal gross appearance and sections from normal parts of atherosclerotic aortae show that no difference in regard to changes related to age can be demonstrated

As the role of the fat in the fibrous intimal changes until now by far

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scopic evaluation cannot be made A microscopic examination must therefore, be made in order to ascertain the nature of the changes

CHEMICAL ANALYSES

The analyses were carried out on dry defatted tissue from the tunica media and the tunica intima separately The medial analyses were performed on 102 samples of thoracic aorta The intimal analyses were performed on 20 aortae 12 of the vessels showed atherosclerosis with distinct fibrous plaques and the analyses were carried out on normal intimal tissue as well as plaques All the analyses were analysed for their statistical significance

The anal a omp f h

charides while the amount of hexosamine indicates the amount of both acid and neutral mucopolysaccharides The concentration of hydroxyproline indicates the collagenous content

If chemical analyses of aortic tissue are to be of any significance at all it is imperative to make the determinations on isolated intimal and medial tissue A brief description of the results is rendered

In the *medial tissue* there is a significant increase in the hexosamine content from birth to 90 years of age (from 1000 to 1600 mg/100 g dry, defatted decalcified tissue) The hydroxyproline concentration varies between 3000 to 3500 mg/100 g of tissue with a slight decrease in the

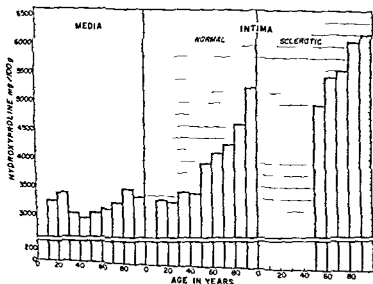


Fig 1

The hydroxyproline content in medial and intimal aortic tissue with age and intimal fibrosis (mg per 100 g of dry defatted decalcified tissue weight)

has been established the author has considered it relevant to advance the following conceptions:

Intima lipidosis meaning an accumulation of lipid in the intima. The lipid accumulates in lumps with increasing age, and these lumps gradually become visible as the so-called *lipoidal plaques*. In the pathology this accumulation of lipid with formation of plaques is often called *atheromatosis*. *The lipoidal plaque is a localized accumulation of* extracellular lipid often surrounded by an abundant amount of "foam cells" peripherally. Deposits of cholesterol crystals may often be seen centrally in the lipoidal plaques of a certain size. Neither cellular nor fibrous reactions are seen in connective tissue surrounding small plaques, but in fairly large plaques with crystal deposits, a superficial intimal fibrosis with bundles of collagenous fibrils running in parallels is often visible. To facilitate a survey the latter fibrosis may be termed a "secondary fibrous plaque" to distinguish it from the fibrous plaque proper.

Intimal fibrosis meaning an accumulation of collagenous or collagen-like fibrils in the intima. In localized areas the fibrils run a parallel course and the tunica intima is converted into a *fibrous plaque*. The transition stage from normal intimal tissue to fibrous plaques is fairly distinct, for the collagenous fragments will—in a rather narrow zone—increase in number and size, gradually forming fibrils running in parallels. Fibrils placed centrally in a fibrous plaque are often seen to increase in thickness and the central parts of these fibres do not take stain. The uncoloured part of the fibrils consists of a homogenous substance, *and where this becomes pronounced the normal* fibrillar structure may be obliterated and a large accumulation of an uncoloured, homogenous tissue—an atheroma—is formed.

These definitions of the lipoidal and fibrous plaques correlate well *with the definition drawn up by the WHO study group (1958).*

Lipoidal plaques are often observable in the aortae without any medial mineralization. On the other hand neither primary nor secondary fibrous plaques are ever seen without a pronounced medial mineralization. Calcium deposits in the intima are observed *solely in connection* with fibrous plaques. The mineralization starts around the collagen fibrils in the ground substance, but increasing deposits of calcium will obliterate the fibrillar structure.

It should be emphasized *that the atherosclerotic process is primarily* localized to the intima.

It is often possible by macroscopic examination of an atherosclerotic aorta to distinguish between the two different forms of plaques. The term *lipoidal plaques* are superficial yellow or yellowish grey intimal lesions which are stained selectively by fat stains and the term *fibrous plaques* are circumscribed, elevated intimal thickening which is firm and grey or pearly white. However, both plaque forms may be complicated by ulceration and calcareous deposits so that a definite *macro-*

DISCUSSION

There is marked correlation between the histochemical and the chemical examinations above mentioned and they confirm the supposition that in the intima as well as in the media an accumulation of complex carbohydrates is occurring. Moreover the depositing of calcium salts in the media seems to be a normal age process, whereas a depositing of calcium in the intima never occurs except in relation to fibrous plaques. The lipid content in the media is relatively scanty, whereas the marked depositing of lipid in normal intimal tissue is an age process to a great extent independent of the formation of fibrous plaques.

As mentioned in the introduction the most common methods for evalutation of the atherosclerotic process in a vessel or a vascular section is a pathological anatomical surface evaluation and/or chemical analyses. In the following these principles will be further discussed.

The most common method by which to evaluate a surface is grading in accordance with the size of the individual plaques or possible complications as calcareous deposits or ulcerations. *Gore & Tejada* (1957) introduced—as some of the first—an evaluation of the total plaque area in relation to the total intimal surface and only during the latest years a few investigators have attempted to distinguish between lipoidal and fibrous plaques.

The above mentioned WHO *study group* (1958), and an American committee for the study of atherosclerosis (*Holman et al* 1960) have simultaneously attempted to set up uniform rules for surface evaluation of the aorta, thus enabling a comparison of the results from various materials. The evaluation is made as follows:

1) Quantitative evaluation	The total plaque area in relation to the total intimal surface
2) Qualitative evaluation	a) Lipoidal plaques
	b) Fibrous plaques
	c) Possible complications as ulceration and/or calcareous deposits

a) b) and c) are subsequently compared with the entire plaque area, which indicates the role of each individual group in the atherosclerosis.

In order to enable a macroscopical distinction between the lipoidal and fibrous plaques the entire intimal surface may be stained with a specific lipoid staining, for instance Sudan III which colours the lipid accumulations clearly. Nevertheless it may often be difficult and now and then impracticable to decide whether a plaque belongs to the lipid or the fibrous type or possibly is a mixture of both. In these cases microscopical examination of each individual plaque is required. The simple macroscopical surface evaluation is naturally extremely subjective and independent evaluations of the same material by several qualified scientists give highly different results (*Gierlsen* 1960). Surface evalua-

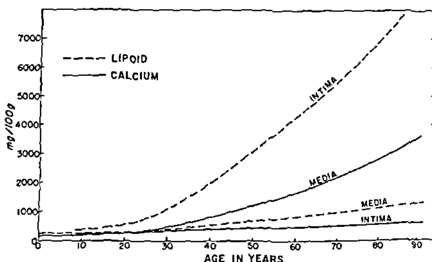


Fig 2

The lipid and calcium concentrations in intimal and medial aortic tissue with normal gross appearance (mg per 100 g of dry defatted tissue weight)

3rd to 5th decade (Fig 1) The hexuronic acid content is constant with advancing age

The calcium concentration in the tunica media shows a distinct increase with age (from 200 to 3500 mg/100 g of dry, defatted tissue), whereas the lipid content is low and only shows a slight increase (from 200 to 1200 mg/100 g of dry, defatted tissue) (Fig 2)

Conditions in the *normal intimal tissue* are quite different The hexosamine content is increasing until about the 5th or 6th decade (from 1100 to 1750 mg/100 g of dry, defattet, decalcified tissue), after which a slight decrease sets in The hydroxyproline content shows a considerable increase with age (from 3300 to 5300 mg 100 g of dry, defatted, decalcified tissue) (Fig 1) There is a slight fall in the hexuronic acid content with age

The calcium content is low and without any observable increase, whereas the lipid concentration increases vigorously with age (from 300 to 8000 mg 100 g of dry, defatted tissue) (Fig 2)

In the *fibrous plaques* the hydroxyproline concentration increases in relation to the content in normal intimal tissue (Fig 1) The hexosamine content shows no correlation with the degree of atherosclerosis, whereas there is a complete disappearance of the hexuronic acid In fibrous plaques without gross calcium salts the concentration of calcium ranges from 400 to 3700 mg 100 g of dry, defatted tissue

Comparisons between the chemical findings in the media and in the intima show that the hexosamine contents in the two layers are almost identical, whereas there are large deviations in the contents of hydroxyproline, calcium salts, and lipids

DISCUSSION

There is marked correlation between the histochemical and the chemical examinations above mentioned and they confirm the supposition that in the intima as well as in the media an accumulation of complex carbohydrates is occurring. Moreover the depositing of calcium salts in the media seems to be a normal age process, whereas a depositing of calcium in the intima never occurs except in relation to fibrous plaques. The lipid content in the media is relatively scanty, whereas the marked depositing of lipid in normal intimal tissue is an age process to a great extent independent of the formation of fibrous plaques.

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[illegible] intimal surface may be stained with a specific lipoid staining, for instance Sudan III which colours the lipid accumulations clearly. Nevertheless it may often be difficult and now and then impracticable to decide whether a plaque belongs to the lipid or the fibrous type or possibly is a mixture of both. In these cases microscopical examination of each individual plaque is required. The simple macroscopical surface evaluation is naturally extremely subjective and independent evaluations of the same material by several qualified scientists give highly different results (*Giertsen* 1960). Surface evalua-

tion without distinguishing between lipid and fibrous plaques must be considered unserviceable for scientific purposes

Numerous chemical examinations of human aortae have proven that in perfectly normal aortic walls there is a considerable increase of lipid with ageing (*Wells* 1933, *Meeker & Lobling* 1934, *Rosenthal* 1934, *Zeek* 1936, *Burger* 1939, *Weinhouse & Hirsch* 1940, *Bruger & Chassin* 1941, *Bjornsson* 1941, *Faber* 1946, *Faber & Lund* 1949, *Buck & Rossiter* 1951, *Hevelke* 1956, 1958, *Buddecke* 1958, *Bottcher et al* 1958, *Kanabrocki et al* 1960) Large amounts of the intimal lipid in an atherosclerotic aorta are thus not included in the atherosclerotic areas for which reason the lipid content does not serve as a particularly useful indicator of the atherosclerotic process

For this reason *Wells* (1933) pointed out that a considerable discrepancy existed between the lipid content in the aorta and its macroscopic status, and in his thesis *Bjornsson* (1941) compared the macroscopical evaluation of the atherosclerotic degree in aortae (lipoidal + fibrous plaques + possible complications) and the amount of cholesterol in the vascular wall, and he demonstrated that there was no relation between the amount of cholesterol and the sclerotic degree These results cannot astonish those who are familiar with the microscopical picture of the lipid distribution in the aortic wall (*Holman et al* 1957, *Zugibe & Brown* 1960, *Bertelsen* 1961a, b)

Fragmentated chemical analyses of the lipid in human aortae partly with increasing age and partly with varying degrees of atherosclerosis show no definite change in the qualitative composition of the lipid (*Meeker & Lobling* 1934, *Zeek* 1936, *Weinhouse & Hirsch* 1940, *Buck & Rossiter* 1951, *Bottcher et al* 1958, *Mead & Gouze* 1961)

Finally it is worth mentioning that *Malinow et al* (1957) after cholesterol feeding of rabbits found no direct relation between the amount of lipid in the aorta and the macroscopically evaluated lipidosis

The presence of lipid in the tunica intima has been a subject of discussion during the last 50–75 years To day it is widely held that lipoproteins are filtered through the intima and resorbed by vasa vasorum in the luminal part of the media It is very likely that this filtration is influenced by numerous factors, and the amount of lipid in the intima will probably be dependent on the speed of the filtration, the thickness of the intima, the resorption etc It is possible that besides the filtration of lipid a synthesis of both cholesterol and phospholipids occurs in the intima but it is not ascertained to what extent the lipidosis is due to this synthesis

It is thus evident that the qualitative and the quantitative lipid examinations hardly are of any value in the grading of atherosclerosis when normal intimal tissue may contain large amounts of lipid while on the other hand relatively small amounts of lipid chiefly may accumulate in plaques

The above mentioned papers by *Weinhouse & Hirsch* (1940) and

Bjornsson (1941) furthermore demonstrated that there was no relation between the amount of calcium in the aorta and its macroscopically evaluated degree of atherosclerosis This correlates perfectly with the fact that the main part of the calcium amount is found in the tunica media independently of the atherosclerotic process in the superficial intima whereas calcium only is found in the intima as a complication to the fibrous plaques

Conclusively it appears that the content of lipid in the aorta *only* expresses the degree of the intimal lipidosis (diffuse lipidosis + possible lipoidal plaques), while the calcium amount first of all expresses the calcium content in the media Therefore, none of these values appear appropriate as an indicator of the degree of vascular atherosclerosis

Normally the intimal tissue contains large amounts of uronic acid while the concentration in the fibrous plaques decreases considerably (*Pernis & Clerici* 1957, *Bertelsen* 1962) The reduction in the amount of uronic acid in the plaques simultaneous with the relatively unaltered amounts of hexosamine indicates that with intimal fibrosis a replacement of acid mucopolysaccharides with neutral takes place which either is found interfibrillarly in the scarce ground substance or is attached to the collagen fibrils *Bertelsen* (1961b) has furthermore found that the collagen fibrils in the fibrous plaques are coloured vigorously with Schiff's reagent after oxidation with periodic acid which supports the assumption that neutral carbohydrates are bound to the fibrils

Sobel et al (1958) have experimented with human skin and demonstrated an increase of the collagenous content, and a decrease of the hexosamine content with increasing age which means, that the ratio hexosamine collagen falls considerably The authors are apt to regard this *ratio as an expression of the age of the connective tissue In aortic* tissue the conditions are somewhat different In the tunica media the collagenous content is relatively constant whereas the amount of hexosamine increases considerably for which reason the ratio hexosamine/collagen must increase with age In normal intimal tissue the ratio is approximately constant until the fifth or sixth decade where it decreases a little, while the ration in fibrous plaques falls considerably compared with normal intimal tissue The intimal changes with age and particularly the formation of fibrous plaques resemble thus the age changes in the skin, which *Yu & Blumenthal* (1958) earlier have suggested

To sum up it is possible to distinguish between the following changes in the aortic wall

a) [illegible]

[illegible] where is considerable amounts of calcium salts are deposited with ageing (apatite-like compounds) In *the tunica intima* a considerable proliferation of the cells takes place simul-

taneous with an accumulation of the ground substance With age a marked lipidosis and an increased collagen content is seen

b) *The specific atherosclerotic changes* are localized to the intima and consist partly of localized lipoidal plaques partly of primary and/or secondary fibrous plaques Calcium salts are only deposited in association with the fibrils in the fibrous plaques but the deposit must be considered a causal complication because far from all fibrous plaques contain them

It is dubious whether it is quite permissible to distinguish between age changes and atherosclerotic changes although the age changes to a certain extent no doubt are responsible or necessary for the development of the atherosclerotic changes

SUMMARY AND CONCLUSION

When defining the atherosclerosis it is necessary to follow the lines of direction worked out by WHO (1958), and to consider the atherosclerosis *as a complex of intimal changes i e an accumulation of ground* substance, lipid and collagen possibly complicated with calcareous deposits A possible mutual dependancy between the above mentioned factors is still unestablished and until further notices appear the lipidosis and the fibrosis in the vessel wall must be considered two individual factors in the atherosclerotic process

When grading the atherosclerotic degree of an aorta it is necessary to distinguish between the lipoidal plaques and the fibrous plaques, and the two different kinds of plaques must be compared with the entire plaque area and the total intimal surface

Chemically the degree of lipidosis and fibrosis is an intimal section may be characterized by its lipid and hydroxyproline content, but it must be emphasized that these values are not directly an expression of the atherosclerotic degree in a vessel Possible complicative calcareous deposits are demonstrated by means of calcium analyses

As the hexuronic acid content in fibrous plaques decreases markedly or disappears completely compared with the content in normal intimal tissue simultaneous with a relatively unaltered hexosamine content and an increase of the hydroxyproline concentration, it is conceivable that the ratio hydroxyproline/hexuronic acid and ratio hydroxyproline/hexosamine may serve as a numerical indicator of the fibrotic degree in intimal tissue

It is, however, necessary to review the chemical grading method thoroughly and for the time being the above mentioned macroscopic and microscopic evaluation of an aorta the most appropriate grading method

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CHROMOSOME ABERRATIONS IN BLOOD, BONE MARROW, AND SKIN FROM A PATIENT WITH ACUTE LEUKAEMIA TREATED WITH 6 MERCAPTOPURINE

By

BENT PEDERSEN

Received 17 xi 63

Recent publications have demonstrated chromosomal aberrations in human and other mammalian cells after treatment with cytotoxics *in vivo* (*Conen & Lansky* 1961) and *in vitro* (*Somers & Hsu* 1962) The aim of this report is to present the results of a chromosomal investiga tion of cells from blood bone marrow and skin of a patient with acute leukaemia under treatment with a cytotoxic agent

CASE REPORT

A 59 year ld male wh in 1950 was treated by resection of the stomach on ac count f a duodenal ulcer was hosp tal zed repeatedly dur ng 1961 and 1962 with a megal blastic anaemia of uncerta n type and treated with blood transfusions l er extract and predn s ne In Decemb er 1962 a bone marrow sample showed 30 5

METHODS AND RESULTS

The leuk cytes were cult vated accord ng to a sl ght mod ficat n of the method f *M rhead et al* (1960) The marr w spec men was not cultured but treated mmed atel after aspirati n acc rd ng to a mo l f a

1 s w rk has been supported by a scholarship from the Lady Tata Memorial Trust and a grant from Anders Hassellalch s Fon l til le ikaemiens l ekæmpelse
I am indebted t pr f *Aa Videbæk* f r kind permission to examine the patient
The expert techn cal assistance of Mrs *B rthe Frost* is gratefully acknowledged

RESULTS

50 mitoses from blood and skin and 40 from bone marrow were counted, and analysed according to the Denver classification by subdivision into 9 groups as shown in Table 2

TABLE 1

Distribution of Chromosome Numbers of 140 Mitoses from Blood Bone Marrow and Skin

	39	40	41	42	43	44	45	46	47	48	Total	Cells with normal karyotype	%
Blood							2	47	1		50	45	90
Marrow		1	1		3	4	2	11	18		40	4	10
Skin	1			1			6	37	2	3	50	33	66
Total	1	1	1	1	3	4	10	95	21	3	140	82	56

Table 1 shows the chromosome numbers of the 3 tissues 18 of the 40 bone marrow mitoses showed 47 chromosomes Thirteen of the 50 mitoses of the skin biopsy material showed abnormal chromosome numbers, ranging from 39 to 48 Table 2 gives more details about the analysed cells from the tissues with 44 to 48 chromosomes The extra chromosome of the bone marrow mitoses with 47 chromosomes was an acrocentric of the 13 15 group in 17 mitoses, and a chromosome of the middle group (6-X-12) in one Seven members of the 13-15 group were also seen in bone marrow mitoses with chromosome numbers other than 47 None was found in the blood culture mitoses, and only one in the skin biopsy material In blood and skin preparations no consistent abnormality was found

In the skin preparations several tetraploid mitoses were seen, whereas the bone marrow preparations showed only one, and none were found in the blood preparations

Three deletions of the chromatid type were found in the examined mitoses of the skin biopsy, and 2 in those of the blood Two chromosome deletions were found in each of these tissues, whereas the bone marrow showed one One chromosome fracture was seen in preparations from skin and one in those from marrow

No Philadelphia chromosomes were seen in any of the 3 tissues

DISCUSSION

Earlier publications have reported a rich variation of chromosome aberrations in blood and bone marrow from patients with acute leukaemia (*Baikie et al* 1961 *Bottura et al* 1961, *Grouchy & Lamy* 1962, *Hungerford & Nowell* 1962 *Sandberg et al* 1962) In some of the cases the picture was characterized by an aberration, which was *common for*

TABLE 2

Supernumerary (+) and Missing ( ) Chromosomes in 51 Mitoses from Blood (B), Bone Marrow (M), and Skin (S) with Chromosome Numbers between 44 and 48

	1			2			3			4 5			6-X 12			13 14 15			17 18			16 19 20			21 22 Y			Total
	B	M	S	B	M	S	B	M	S	B	M	S	B	M	S	B	M	S	B	M	S	B	M	S	B	M	S	
44 +								–			–	–	–				2		–	–	–	–	–	–	–	–	–	2
								1						5				–	–	–	–	–	2	–	–	2	–	10
45 +						–								–			1	–	–	–		–	–		–	–	–	1
									–					3		–	–	2	1	1	–	1	–	–	–	–	3	11
46 +				–	–				1		–		2	1			7	1				–		–	–	–	1	13
			1	–		–	1	1	–					3	2			–	–		–	–	1		1	2	1	13
47 +	–							1			–			1	2	–	17		–	–	–	2	–	–	–	–	–	23
		1		–	–		–		–		–			–		–	–	–	1	–	–	–	–	–	–	–	–	2
48 +			–			–		–		–				–	6			–		–		–		–	–	–	–	6
		–					–			–	–	–			–			–	–	–	–	–	–	–	–	–	–	–
Total		1	1		–		1	3	1	–			2	13	10		27	3	2	1	–	3	3	–	1	4	5	81



Fig 1
The karyotype of a bone marrow mitosis with 47 chromosomes and 7 members of the 13 15 group

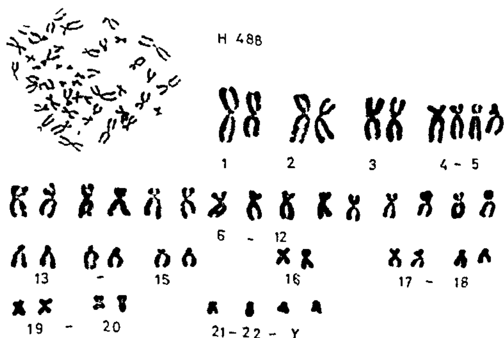


Fig 2
A karyotype from skin culture with 45 chromosomes and only 4 members of the small acrocentric group

a major proportion of the examined cells, indicating that these cells belonged to the same strain, but few patients have yet shown the same karyotypical picture In other cases, again the aberrations have varied from cell to cell without any common feature In the present case a strain with 47 chromosomes and 7 members of the 13–15 group was found in the marrow It is possible that these cells are myeloblasts, which at the time when blood and bone marrow were obtained for examination were numerous in the marrow and rare in the blood

The aberrations found in the blood and skin preparations showed no consistent feature It is difficult to exclude that these aberrations are artefacts Although ruptured cells have been avoided, and only cells with a regular circumference were chosen during the selection of mitoses for analysis, chromosomes may have been lost in preparation Very often homologous chromosomes differ in size or centromere position Such conditions seem to be caused by a different degree of distension of the chromosomes on the slide Asymmetrical distension may change the centromere position of the chromosome If the material is stained with Giemsa, the investigator is often warned by a slightly lighter staining of the distended chromosomes As far as possible these pitfalls have been avoided In all cases with the slightest possibility of an error caused by distension, the aberration was not registered as such

Earlier investigations have shown that nitrogen mustard can induce chromatid and chromosome deletions, fractures, and fusions *in vivo* (*Conen & Lansky* 1961) Similar lesions have been induced *in vitro* by hydroxylamine (*Somers & Hsu* 1962) and radioactive nucleosides (*Bender et al* 1962), and both *in vivo* and *in vitro* by X-rays (*Bender & Gooch* 1962, *Buckton et al* 1962, *Chu et al* 1961, *Lindsten* 1959). In addition to these lesions, *Buckton et al* (1962) found, in blood from irradiated patients, cells with 46 chromosomes but an abnormal distribution of chromosomes amongst the subgroups These latter abnormalities seemed to persist, in contrast to deletions and fragments, which disappeared rather rapidly after the irradiation

In the present case no sign of leukaemic infiltrations was seen in the skin The chromosal aberrations found in the biopsy are therefore not likely to be leukaemic At the time when the biopsy was obtained the patient had been treated with 6-mercaptopurine for 21 days It therefore seems probable that the aberrations were induced by the cytotoxic agent

Chromosomal aberrations have been described in untreated cases of acute leukaemia (*Hungerford & Nowell* 1962) It therefore seems probable that leukaemic and cytotoxic factors are each playing a part in the causation of the karyotypical abnormalities, which are detected in blood and bone marrow from patients with acute leukaemia, treated with cytotoxic agents such as 6-mercaptopurine

The predominant abnormal features of leukaemic cells are aneuploidy and an abnormal distribution of chromosomes among the subgroups

Possibly some chromosomes are more vital to the cells than others Absence or excess of such chromosomes may then be fatal and therefore never seen on karyotypical investigations Absence or excess of other chromosomes might on the other hand be advantageous to the cell under certain circumstances and consequently appear with great frequency In the present case a supernumerary member of the 13–15 group could be advantageous in the bone marrow In the mitoses of the skin biopsy a small acrocentric (21–22–Y) is missing in three and a large acrocentric (13–15) in two cells Two cells show a supernumerary member of the middle group (6-X-12), and three cells contain two supernumerary members of the middle group As the frequency of these cell types is moderate, the mentioned aberrations do not seem to be very advantageous, nor can they be very harmful Possibly a broad investigation of patients with acute leukaemia show a rather limited total number of different aberrations

Abnormal distributions of chromosomes among the subgroups in mitoses with 46 chromosomes may be caused by translocation processes

SUMMARY

Chromosomal examination of blood, bone marrow, and skin has been performed in a patient with acute leukaemia after treatment with 6-mercaptopurine for a few weeks All mitoses have been analysed according to the Denver classification, and the results are presented Besides other abnormalities the bone marrow showed a strain with 47 chromosomes and 7 members of the 13 15 group In the blood and skin a variety of abnormalities, without any constant features, was found The aberrations detected in mitoses of the skin biopsy material are thought to have been induced by the cytotoxic therapy

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STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

4 *The Question of Immunity in Adoptively Immunized Virus Carriers*

By

MOGENS VOLKERT, JØRGEN HANNOVER LARSEN and CHARLES J PFAU[1]

Received 26 xi 63

In the foregoing reports (21, 22, 23) experiments were described which showed that a responsiveness to the LCM virus in virus carriers could be adoptively conferred by transplantation of lymphoid cells from immune donors This conferred responsiveness had many similarities to the immunity to skin and tumour grafts which also can be conferred by transplantation of immunologically competent cells The two phenomena might therefore be caused by the same basic immune mechanism However, up to now, the only assay used to estimate a responsiveness conferred on virus carriers has been a determination of virus titre reduction For this reason some doubt must still prevail concerning the rôle of the immunity in the transplanted virus carriers

It is the purpose of this article to describe further studies on the immunity problem in virus carriers transplanted with immune lymphoid cells

MATERIAL AND METHODS

Complement fixing antigens were prepared from brains of infected baby mice by the method described by *Grešiková* & *Casals* (5) and by *Clarke* & *Casals* (2) Antigen preparations with titres of 16 were usually obtained

Complement fixing control antigen Extracts of normal brains from baby mice were prepared in the same manner as for the infected brains

Complement fixation test Each serum was tested against the virus antigen and the control antigen the latter being used in the same concentration as the virus antigen To determine the serum titre 0 1 ml of antigen containing four units was added to 0 1 ml amounts of inactivated serum in two fold dilutions To each tube two units of complement in 0 1 ml were then added and the mixtures were kept at 4° C overnight The following morning the haemolytic system (2 units of haemolysin in 0 1 ml plus 0 1 ml of 2 per cent washed sheep red blood cells) was added and the tubes placed in a water bath at 37° C for one hour Readings were made after one additional hour at room temperature The endpoint was taken as the last tube with at least 50 per cent complement fixation

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1 Postdoctoral Fellow of the U S Public Health Service

The authors are indebted to Dr *A Paucker* (Philadelphia U S A) for kindly providing the Vesicular Stomatitis Virus and to *Mrs Højer Simonsen* *Miss I Halsted* and *Miss Dyrmig Petersen* for skilled technical assistance

Virus neutralization test This was performed with ten fold serial dilutions of virus against undiluted serum 10 per cent suspensions of infected mouse spleens served as the virus source The virus was diluted in PBS plus 10 per cent inactivated rabbit serum The serum virus mixtures were incubated as indicated below and subsequently inoculated intracerebrally in amounts of 0 03 ml into white Swiss mice weighing 12 14 grammes The observation time was 14 days The LD_{50} and the logarithmic neutralization index were calculated according to the method of Reed and Muench

The interferon assay was essentially as described by *Paucker* (12) The tissue cultures were primary cultures of fibroblasts from 14 day old mouse embryos grown in the media used by either *Salk et al* (14) or *Eagle* (4) The test virus was the Indiana strain of Vesicular Stomatitis virus

Interferon preparations Control preparations were made from influenza virus (strain PR 8) infected embryonated eggs according to the procedure of *Wagner* (24) and stored at —25° C Clarified allantoic fluid was thawed adjusted to pH 2 with $HClO_4$ and after standing for four hours at 4° C centrifuged at 700 × G for 30 minutes The supernatant (readjusted to pH 7 to 8) or the precipitate formed after [illegible]

[illegible]

genizer for 15 seconds at full speed The remaining steps of the extraction procedure were exactly as described above except the fluids were recentrifuged immediately after adjusting to pH 2 Some of the samples were furthermore concentrated by pervaporation in Visking tubing before being tested

The virus strains of mice general experimental conditions and all the methods not mentioned above were as described in the foregoing papers (22 23)

EXPERIMENTAL

Virus

Recently *Shope* (15) and *Herriott* (8) have put forward the hypothesis that naked infectious nucleic acids might play a rôle in certain latent virus infections For this reason the characteristics of the virus in LCM virus carrier mice were investigated

Centrifugation experiments Preliminary experiments had shown that the LCM virus could be sedimented by centrifugation at about 100 000 × G for one hour As such a centrifugation cannot sediment nucleic acid molecules it should be possible by centrifugation procedures to get results which might indicate whether the virus in carriers was particulate or not The following two experiments were therefore carried out Two separate 10 per cent spleen suspensions in Hanks balanced salt solution were prepared from virus carriers [illegible]

[illegible] no 40 for [illegible] the super[illegible] preparations were collected and titrated for virus Comparison of the titres before and after the final centrifugation in two experiments is shown in Table 1

Blood was also collected from both the virus carriers and the normally infected mice and pooled separately Just enough heparin was added to the blood to prevent clotting After centrifugation for 20 minutes at 1500 × G the plasma was collected and centrifuged in the

TABLE 1
Virus Characteristics

Virus	Sedimentation 100000 × G 1 hour		Neutralization 4 °C 1 hour		Treatment with DNase and RNase	
	before	after	normal serum*	immune serum*	before	after
10 per cent spleen susp from	8 3	3 5	6 5	2 4	7 9	7 9
carrier mice	8 5	3 0	6 9	<2 0	6 9	7 5
10 per cent spleen susp from	5 8	<1 0	6 1	1 5	3 0	2 8
normally infected mice	6 8	1 0	5 5	<2 0	4 0	4 1

* Guinea pig serum

Spinco, rotor no 40, for one hour at 74,000 × G After centrifugation the supernatants were collected and titrated for virus A comparison of the plasma titres before and after the final centrifugation showed clearly that there is no difference between the ability of the virus from virus carriers and from normally infected mice to be sedimented

Enzyme studies

Free infectious nucleic acids should be destroyed by nucleases, whereas a protein-coated virus particle should not be influenced by these enzymes For this reason, experiments were carried out to determine whether the virus in the virus carriers could be inactivated by nucleic acid destroying enzymes Pools of 10 per cent spleen suspensions from virus carriers were selected as virus sources To the virus preparations were added DNase and RNase to a final concentration of 250 γ each, and Mg^{++} to 0 001 M (The nucleases were from Worthington Biochem Corp , New Jersey) The virus-nuclease mixtures were kept at 4° C for one hour and then titrated for virus Representative results are recorded in Table 1 It will be seen that no titre reduction is caused by the enzyme treatment In order to test whether the enzymes were active in our system, a control experiment was carried out Commercial preparations of DNA and RNA plus the above-mentioned amounts of nucleases and Mg^{++} were added separately to the spleen suspension After one hour at 4° C the amount of intact nucleic acids was determined by the acid precipitation method described by *Conolly, Herriott & Gupta* (3) The results indicated full activity of the added enzymes

Antigenicity Traub & Schafer (18) has already described an CF antigen prepared from the spleens of virus carrier mice Recently *Grešíková & Casals* (5) succeeded in preparing a potent CF antigen from the brains of young mice infected shortly after birth Using Casals' method, we have prepared antigens from the brains of virus carrier mice and found that these antigens react with immune serum from ordinary immune mice Moreover, we have found repeatedly in virus neutralization experiments that guinea pig hyperimmune serum neutralizes virus from

virus carriers and from normally infected mice equally well These results would indicate that the virus in virus carriers has not lost its antigenic character

Conclusion The virus studies have not revealed any peculiarity with the virus in virus carrier mice

Antibodies in LCM Virus Infection

For detecting antibodies to the LCM virus only the complement fixation test and the neutralization test have been described Concerning the complement fixing antibodies all parties working with LCM virus infections seem to agree that these antibodies develop in all mature animals infected with LCM virus However when the virus neutralizing antibodies are concerned there is a striking difference between the mouse and all the other species tested In man guinea pigs rabbits and many other mammals neutralizing antibodies are easily demonstrable in the blood after an infection However this is not the case with the mouse Some authors (13 20) have claimed that traces of neutralizing antibodies could be found in hyperimmunized mice but most other workers have failed to detect any (6 16 17)

Our findings are similar to those just mentioned We have also seen complement fixing antibodies develop in both guinea pigs and mice after sublethal virus infections (Fig 1) Titres of about 64 were most common In immune guinea pig serum the presence of neutralizing antibodies was also easy to demonstrate After one hour s incubation of immune guinea pig serum and virus mixtures at 37° C the virus was neutralized In serum from hyperimmune animals (three virus injections through five weeks) log neutralization indexes of 4 5 were

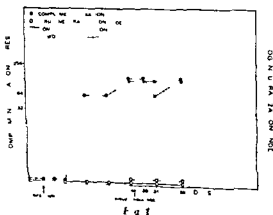


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Conclusion. The virus studies have not revealed any peculiarity with the virus in virus carrier mice.

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For detecting antibodies to the LCM virus only the complement fixation test and the neutralization test have been described. Concerning the complement fixing antibodies, all parties working with LCM virus infections seem to agree that these antibodies develop in all mature animals infected with LCM virus. However, when the virus neutralizing antibodies are concerned there is a striking difference between the mouse and all the other species tested. In man, guinea pigs, rabbits and many other mammals, neutralizing antibodies are easily demonstrable in the blood after an infection. However, this is not the case with the mouse. Some authors (13, 20) have claimed that traces of neutralizing antibodies could be found in hyperimmunized mice, but most other workers have failed to detect any (6, 16, 17).

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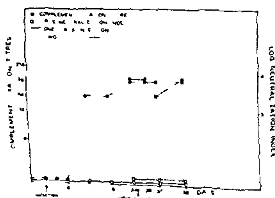


Fig 1

Complement fixation titres and [illegible] mice following intraper[illegible] received 10[illegible] i.c. LD_{50} [illegible]

the rule In mice, however, we failed completely to detect any neutralizing antibodies Blood was tested at different intervals after one or after two to three sublethal virus injections (Fig 1) Blood from mice which had received up to seven virus doses intraperitoneally through a period of 14 weeks was also examined Undiluted serum was incubated with virus for one, two and four hours at 37° C, and for up to 18 hours at 4° C Both fresh and inactivated serum were employed In none of our experiments was it possible to demonstrate even the slightest trace of neutralizing substances in the blood of the immune mice

Blood samples from untreated virus carriers have also been examined, but it was not possible to detect the presence of either complement-fixing or neutralizing antibodies in any of them

Complement-Fixing Antibodies in Virus Carriers Transplanted with Lymphoid Cells from Immune Donors

A group of 45 female C3H 6–8 week old virus carriers were selected for this experiment The blood of each mouse was tested for virus All were positive, and the lowest titre found was $10^{2.4}$ Several pools of blood from these mice were furthermore examined for complement-fixing antibodies, but as usual with this kind of blood all tests were negative in serum dilutions of 2 or above After the preliminary examinations, each mouse in a group of 38 of the selected virus carriers was transplanted with 100×10^{6} lymphoid cells from female donors hyperimmunized by several sublethal doses of live virus (seven injections of 100 i c LD_{50} virus doses intraperitoneally during three months) Seven virus carriers were left untreated and served as controls Five of the transplanted mice were killed on each of the following days 1, 2, 4, 7, 13, 21, and 35 The heart blood was collected from each mouse and titrated for virus Moreover, the blood was pooled for each of the groups of five mice and the pools titrated for complement-fixing antibodies The results are recorded in Fig 2 It will be seen that the mean virus titre curve follows the same pattern as that usually seen after an adoptive immunization with this type and amount of cells During the first four days after the transplantation no depression of the virus titres can be seen, but from then onwards the titre curve drops abruptly On the tenth day after the transplantation the virus titre reduction which had occurred was more than three logs, and a few days later the titres stabilized at a very low level Concerning the complement fixation tests, no antibodies could be detected the first day after the transplantation However, on the second day a small amount was present and from then onwards a steep rise in antibody occurred On the sixth day the titre in the blood of the transplanted mice was higher than 64, which was the titre found in the pooled donor blood The rise in antibody continued and reached as high a titre as 8192 on the 13th day During the following week the titre showed a four-fold decrease, but from then on it seemed to stabilize

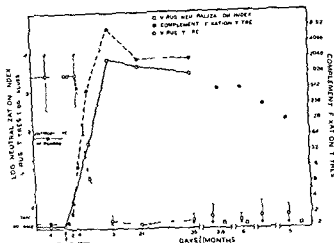


Fig 2

Virus titres and antibody titres of the blood from virus carrier C3H mice before and after transplantation of 100 × 10⁶ lymphoid cells from immune donors

In the control mice the virus titres of the blood remained above 10⁷·⁵ and antibodies were never detectable

In this first experiment, the mice were not observed for more than 35 days However, in other groups of mice transplanted in the same way three and a half months, six months, 10 months and 15 months previously, the blood was titrated for both virus and complement-fixing antibodies The results of these titrations are also recorded in Fig 2 As usual some, but only extremely small quantities, of virus were found in the blood, but the antibody titres were high Even in the group of mice transplanted 15 months previously, the antibody titre found was higher than that usually seen in ordinary mice hyperimmunized by many injections of live virus Repetitions of the experiment just described have given very similar results

Virus Neutralizing Antibodies in Transplanted Virus Carriers

As mentioned above, we had failed to detect virus neutralizing antibodies in the blood of ordinary immunized mice However, the extremely high complement-fixing antibody titres obtained in the transplanted virus carriers made it tempting to investigate the blood of these mice for the presence of neutralizing antibodies

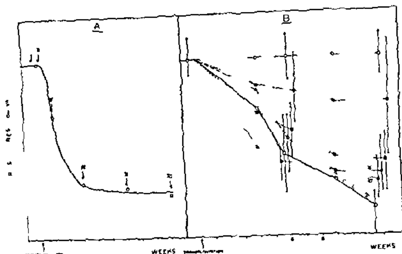
First three serum pools were investigated in a screening test The blood for these pools was taken from C3H virus carrier mice on the 15th to 21st day after the carriers had been transplanted with 100 × 10⁶ isologous lymphoid cells from immune donors All three serum samples were incubated with the virus for one hour at 37° C Two serum pools from normal mice served as controls The results were clear cut All three sera from the transplanted virus carriers contained neutralizing

the rule In mice, however, we failed completely to detect any neutralizing antibodies Blood was tested at different intervals after one or after two to three sublethal virus injections (Fig 1) Blood from mice which had received up to seven virus doses intraperitoneally through a period of 14 weeks was also examined Undiluted serum was incubated with virus for one, two and four hours at 37° C, and for up to 18 hours at 4° C Both fresh and inactivated serum were employed In none of our experiments was it possible to demonstrate even the slightest trace of neutralizing substances in the blood of the immune mice

Blood samples from untreated virus carriers have also been examined, but it was not possible to detect the presence of either complement fixing or neutralizing antibodies in any of them

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A group of 45 female C_3H 6–8 week old virus carriers were selected for this experiment The blood of each mouse was tested for virus All were positive, and the lowest titre found was $10^{2.5}$ Several pools of blood from these mice were furthermore examined for complement fixing antibodies, but as usual with this kind of blood all tests were negative in serum dilutions of 2 or above After the preliminary examinations, each mouse in a group of 38 of the selected virus carriers was transplanted with 100×10^6 lymphoid cells from female donors hyperimmunized by several sublethal doses of live virus (seven injections of 100 i c LD_{50} virus doses intraperitoneally during three months) Seven virus carriers were left untreated and served as controls Five of the transplanted mice were killed on each of the following days 1, 2, 4, 7, 13, 21, and 35 The heart blood was collected from each mouse and titrated for virus Moreover, the blood was pooled for each of the groups of five mice and the pools titrated for complement-fixing antibodies The results are recorded in Fig 2 It will be seen that the mean virus titre curve follows the same pattern as that usually seen after an adoptive immunization with this type and amount of cells During the first four days after the transplantation no depression of the virus titres can be seen, but from then onwards the titre curve drops abruptly On the tenth day after the transplantation the virus titre reduction which had occurred was more than three logs and a few days later the titres stabilized at a very low level Concerning the complement fixation tests, no antibodies could be detected the first day after the transplantation However, on the second day a small amount was present and from then onwards a steep rise in antibody occurred On the sixth day the titre in the blood of the transplanted mice was higher than 64, which was the titre found in the pooled donor blood The rise in antibody continued and reached as high a titre as 8192 on the 13th day During the following week the titre showed a four-fold decrease, but from then on it seemed to stabilize



F g 3A an l B

I g 3A M n

F g 3B Mean v rus titres of the l lood of AkA virus carr er m ce follo ing trans plantat on of 100 × 10⁶ lymphoid cells from the groups of transplanted *mice in experiment 4*

w is injected into e ich of seven mice in a new fresh virus carrier group *When the mice in the* primarily transplanted group were killed the heart blood from each mouse was collected and titrated for virus The me in titre *curve for these* titrations is recorded in Fig 3A The mice in the seco ndarily transplanted groups were bled at intervals and the l l od virus titres determined for each individual mouse The mean titre curve for e ich of these groups is recorded in Fig 3B As controls spleen and lymph node cells were *also harvested* and pooled from the remain *ing* seven mice in the first group *e g* mice which had not been injected with immune cells Amounts *of 100 × 10⁶ cells* per mouse from this pool were transplanted to new virus carriers The mean titre curve following this transpl intation is also recorded in Fig 3B

As *will be* seen from Fig 3A the results of the primary transplanta ti n were as usually found and in three weeks the mean blood virus titre had reached a stabilized low level of about 10⁰ ⁹ The data presented in Fig 3B shows that the transplantation immunity can be secondarily conferred Already 24 hours after virus carrier mice have been trans planted spleen and lymph node cells harvested from these mice and transplanted to other *carriers* have some effect on the virus in the new hosts but great individual variations are apparent indicating a low pot ncy of the transplanted *cells* However cells harvested one week later cause a marked decline in the virus titres of all the new recipients a decline which brings the virus titre level as far down as that seen in

antibodies The titration endpoints were not reached, but in all cases the log neutralization index was higher than three In view of these results, the stored serum samples from the transplanted virus carriers in the experiment described in the foregoing paragraph were titrated for neutralizing antibodies The serum-virus mixtures were incubated at 37° C for one hour Two normal mouse serum pools were included as controls The results recorded in Fig 2 confirmed that neutralizing antibodies are produced in transplanted virus carriers As apparent from the figure, the neutralization antibody curve follows closely the complement-fixing antibody curve, reaching a maximum on about the 13th day after the transplantation From then on it declines slowly Moreover, not only are neutralizing antibodies produced, but the log neutralization indexes obtained are very high

Because of the fact that we had used stored serum in the neutralization tests just described, a new experiment was carried out In this la C_3H virus carriers were transplanted with lymphoid cells from immunized donors On the fifth day, tenth and fifteenth day after the transplantation the mice were bled and the fresh blood titrated for virus complement-fixing antibodies and neutralizing antibodies The results were very similar to those obtained in the foregoing experiment

Other groups of C_3H mice transplanted with 100×10^6 immune lymphoid cells 3½, 6, 11 and 15 months previously were bled separately and the blood tested for virus neutralizing antibodies The results are recorded in Fig 2 It is apparent that already 3½ months after transplantation all measurable neutralizing antibodies had disappeared Thus production of these antibodies does not last nearly as long as the production of complement fixing antibodies

It is also apparent from Fig 2 that with the technique used infectious virus and neutralizing antibodies can be found in the same blood sample

Secondary Transfer of Transplantation Immunity

The fact that the transplantation of immune lymphoid cells to virus carriers had a depressing effect of long duration on the virus and furthermore caused a prolonged antibody production, indicates a long lasting active state of the immunological competent cells in the transplanted mice For the purpose of elucidating this problem further the effect of secondary transfer of the transplantation immunity was investigated

Thirty-five female AKA virus carriers of the same age were selected for the experiment Twenty eight of these were transplanted individually with 100×10 immune lymphoid cells from female donors vaccinated several times All the cells were from the same cell pool and were transplanted to the carriers on the same day One day, one week, three weeks and nine weeks after the transplantation, groups of seven mice were killed and the spleen and lymph node cells harvested A cell pool was made for each mouse group and from this a dose of 100×10^6 cells

the primary transplantation experiments. Lymphoid cells harvested when the decline of virus titre was complete, e g three weeks after the transplantation, can also confer a good immunity, but the effect of these cells is not significantly better than that obtained with the cells harvested two weeks previously. Cells harvested nine weeks after the transplantation, at a time when the low virus titres have been constant for six weeks, were likewise found to have a good effect on the virus in the new recipients The immunity conferred was equal to or perhaps even better than when cells harvested one or three weeks after the transplantation were used

As is also apparent from Fig 3B, lymphoid cells from untreated virus carriers had no ability to confer any immunity

A similar experiment to the one just described was carried out with C3H mice From virus carriers transplanted with lymphoid cells from donors vaccinated several times, secondary transplantations were carried out with cells harvested one day, three weeks and nine weeks after the primary transplantation had taken place The results obtained with cells taken from virus carriers three weeks and nine weeks after a transplantation with 100×10^6 immune lymphoid cells were almost identical to those seen with the AKA mice, i e these cells conferred a good immunity on all the new recipients However, in the C3H experiment, the cells taken only one day after the transplantation did not confer immunity on any of the virus carrier mice to which they were transplanted.

Taken together, these experiments show clearly that the immunity conferred by lymphoid cells harvested from transplanted virus carriers one day after the mice had received an effective transplantation dose, is very weak at its best and often not even demonstrable However, cells harvested one week later can confer a good immunity on the new hosts This immune quality of the spleen and lymph node cells from the transplanted virus carriers remains unaltered for at least two months

Interferon

In discussion of our experiments, it was suggested that the transplantation effect on the virus might be due to interferon produced by the transplanted cells Even in view of all the data which indicated that an immune mechanism was operating, it could not be excluded beforehand that interferon might not play a rôle Experiments were therefore carried out to elucidate this possibility

In the first series of experiments, virus carriers transplanted with 100×10^6 immune lymphoid cells were killed at a time when the tranplantation effect on the virus had reached its maximum The organs were removed and extracts prepared by one of the methods described in the "Material and Methods" section of this paper Each extract was then tested for interferon activity A positive control interferon pre-

paration was included in each test series Table 2 represents a typical experiment, and shows clearly that no interferon activity could be demonstrated in any of our organ preparations These results have been obtained both with two series of organ preparations from frozen organs and two series with freshly harvested tissue The effect of the zinc acetate method (11) of precipitating interferon was confirmed using allantoic fluid from influenza PR8 virus infected embryonated eggs, but gave completely negative results with all the organ extracts Concentration of organ extracts by pervaporation also gave negative results

TABLE 2

Interferon Assay With Organ Extracts from Transplanted Virus Carriers

Organ preparations	Dilution	Plaque counts
Brain	1/2	44
Spleen	1/2	36
Kidney	1/2	50
Liver	1/2	41
Heart	1/2	40
Virus control		45
Untreated cultures		0
Interferon control	1/64	19

In the second series of experiments yellow fever virus strain 17 D (the vaccine strain from the
intracerebral inoculations in
mice and 3) virus carrier A
100 × 10^6 immune lymphoid cells six weeks previously The yellow *fever virus titres obtained* were $10^{7.7}$ in normal mice, $10^{6.0}$ in virus carriers and 10^{5} in the transplanted virus carriers According to *Isaacs (10)* the yellow fever virus production is susceptible to interferon Therefore the equally high titres obtained in the transplanted virus carriers and in the other mice give no support for the assumption of an interferon production caused by the transplanted cells at least not in the brains of the transplanted mice

DISCUSSION

In accordance with many other workers we have failed to detect any antibody production in untreated carrier mice Moreover, the virus studies presented here indicate clearly that virus in carriers can be sedimented easily and is not affected by nucleases, but is neutralized by specific immune serum and can produce a complement fixing antigen The virus therefore, is *apparently a "normal" virus and the absence of detectable immune reaction* in the virus carriers is probably due to an *unresponsiveness of the host*

As described previously (21, 22 23), when virus carriers are trans

the primary transplantation experiments Lymphoid cells harvested when the decline of virus titre was complete, *e g* three weeks after the transplantation, can also confer a good immunity, but the effect of these cells is not significantly better than that obtained with the cells harvested two weeks previously. Cells harvested nine weeks after the transplantation, at a time when the low virus titres have been constant for six weeks, were likewise found to have a good effect on the virus in the new recipients The immunity conferred was equal to or perhaps even better than when cells harvested one or three weeks after the transplantation were used.

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and moreover that the virus is never completely eliminated (23) An antigen stimulus is, therefore, *continuously present*

The permanently active state of the immunological competent cells [illegible] apparent *from the data obtained*
[illegible]
cells from these transplanted mice could [illegible] a good [illegible] new untreated virus carrier mice It is noteworthy, however, that more than a day must elapse before a cell harvest with a good activity can be obtained from the spleen and lymph nodes of transplanted mice The *most simple explanation* of this phenomenon seems to be that only relatively few of the activated, immunologically competent cells may *settle in the spleen and lymph* nodes of the recipients and therefore the cells must multiply before a sufficient amount can be regained by *our method Another possibility is that for many* cells it might take more than a day to travel from the peritoneal cavity to the organs and one has to await their arrival *Other hypotheses can, however, also be* offered

The data presented concerning interferon has failed to give any evidence of the presence of this substance in the organs of transplanted virus carriers Nevertheless it is generally agreed that interferon extraction from organs often does not succeed even in cases where interferon is presumably present Therefore, the negative results with organ extractions do not exclude that interferon might play a role also in our system However the fact that the transplanted virus carriers showed no increase in their resistance to yellow fever virus supports the assumption that interferon is not produced by the transplanted cells Moreover the extremely strong immune reaction caused by the transplanted lymphoid cells seems to be all the explanation needed for the virus reduction Therefore, *until possible concrete evidence of an interferon* action is obtained, we consider it reasonable to assume that it is without importance *for the transplantation effect on the virus*

Probably, the feature observed in our experiments is a change from a state *of passive (tolerant) immunity* and high viraemia to a state of active immunity and low viraemia *Traub* (19) has shown that the first *of* these situations occurs in nature The experimentally produced second situation is of course, highly artificial However, data to be presented *in a* subsequent article indicates that a long lasting state of active immunity connected with persistence of small amounts of virus is often (if not always) brought about in the laboratory as a result of ordinary infections of normal (non tolerant), mature mice Therefore this situation probably also occurs in nature

The demonstration of the simultaneous rise in antibody titre and decrease in virus titre might indicate that the antibodies cause the virus reduction However further proof is needed for this assumption and experiments concerning this problem are in progress in this laboratory

planted with activated lymphoid cells a responsiveness is conferred on the recipients, thus causing a sharp reduction of the virus titres in blood and organs. As shown in the foregoing papers (22, 23), this conferred reactivity is probably an immune reaction. More direct proof for this assumption is presented in this article The experimental data shows clearly that shortly after virus carriers are transplanted with lymphoid cells from immune isologous donors, complement-fixing antibodies occur in the blood These antibodies reach extremely high titres during the first week. Furthermore, not only complement-fixing antibodies are produced in the transplanted mice, but virus neutralizing antibodies can also be found It seems beyond doubt, therefore, that an immune *reaction is provoked However, this* immune reaction is *extraordinarily* strong The complement-fixing antibodies reach a titre level in the transplanted carriers which is more than 100 times higher than that usually seen even in hyperimmunized normal mice, and in the experiments presented in this paper more than 100 times higher than in the donors Moreover, as mentioned above, virus-neutralizing antibodies are also produced, and this kind of antibody has never been detected even in the most hyperimmunized mice What makes this strong immune reaction even more striking is the fact that the cell doses which produce it contain less than half the cells which can be obtained from one single donor, *i e* it is probably not more than roughly a fourth of all the lymphoid cells one donor contains The reason for the extremely strong reactivity of the transplanted cells is probably the effect of an enormous antigen stimulus The virus in the virus carriers reaches titres 10^3 to 10^4 in the blood, and of more than 10^8 in certain organs, and therefore the transplanted cells are practically bathed in virus antigen However, this strong stimulus does not seem to kill the cells or to exhaust them The antibody titre is kept at a high level for weeks The very early initiation of the antibody production and the titre level obtained indicate a "secondary response"

Our data has also shown that the immunity conferred on virus carriers by transplantation of activated lymphoid cells is of extremely long duration More than a year after a transplantation, antibody titres are found which are about four times higher than those originally measured in the donor blood *Chase* (1) reported in 1950 that transfer of lymph node and spleen cells from highly sensitized guinea pigs to normal animals led to the development of anaphylactogenic antibodies in the recipients Since then, *Harris & Harris et al* (7) and many others have also shown that transplanted activated lymphoid cells cause an antibody production in the new host However, most of these authors have used either non-tolerant animals or a homologous donor-host system, and in such experiments the antibody titres decrease and disappear rather quickly The reason for the permanent high antibody production in the transplanted virus carriers might be that in our isologous system no host versus graft reaction operates against the transplanted cells,

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... ent fixing antibodies persist for at least 15 months while

ansplanted
lymphoid cells can be secondarily conferred on other tolerant virus carriers by means of the spleen and lymph node cells from the primarily transplanted mice

No evidence has been obtained which would indicate that interferon plays any role in the virus reduction in transplanted virus carriers

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It is an interesting observation that both infectious virus and complement-fixing antibodies as well as neutralizing antibodies can be present in the same blood sample.

As mentioned in a foregoing paper (22), the transplantation of virus carriers with activated lymphoid cells has usually been a harmless procedure. In the majority of the experiments, even the highest cell doses, corresponding to all the spleen and lymph node cells which can be obtained from 10 donors (i.e. 2000×10^6) have caused no sign of clinical disease in the recipients. As we now know that the transplantations cause an extraordinarily strong and acute immune response, this observation speaks against the assumption of *Rowe* (13) and *Hotchin* (9) that an immunological response to LCM virus in mice should cause a clinical illness.

On very rare occasions we have observed illness in transplanted virus carriers, but only in AKA mice, and not in C3H mice. The AKA strain of mice has a high incidence of leukaemia while the C3H mice are free from that disease. Moreover, the harmful effect of transplantation has been associated with certain cell batches only, and occurred in such a way that all the mice which received cells from these batches became ill and often died. No correlation between cell doses and disease has been found. Therefore, we consider it most likely that the illness seen has been due to incidental transfer of leukaemic cells and virus and is not caused by the responsiveness conferred.

Hotchin (9) has also reported that his virus carrier mice often acquired a wasting disease and died at the age of about one year. We have never observed anything of that kind, either in AKA mice or in C3H mice. On the contrary, our virus carriers live as long and are as healthy as the controls.

SUMMARY

No difference has been observed between the LCM virus in neonatally infected virus carriers and the virus from ordinary infected mice by any of the following procedures: Centrifugation, treatment with DNase and RNase, inactivation by specific immune serum. Moreover, neither complement-fixing nor neutralizing antibodies have been detected in virus carriers. These results support the hypothesis that the LCM virus carrier state is caused by the tolerance phenomenon.

By transplantation of lymphoid cells from immune donors the non-responsive, tolerant state of immunity is changed to an active state. As a result both complement-fixing and virus neutralizing antibodies are produced. Moreover, the balance between the host and the virus is changed from a state with high virus titres in blood and organs to one where the virus is hardly detectable.

The antibody response induced by transplantation in tolerant virus carriers is extremely strong. This is indicated by the fact that the complement-fixing antibody titres may be 100 times higher than those usu-

ally seen in ordinary immune mice and moreover that high titre neutralizing antibodies are also produced

The complement fixing antibodies persist for at least 15 months while the neutralizing antibodies disappear in about three months

Th.. .m. n.ly conferred on tolerant virus carriers by transplanted

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The University of Bergen School of Medicine the Gade Institute
Department of Microbiology Bergen Norway

SEROLOGICAL TYPING OF *STAPHYLOCOCCUS AUREUS*

Factor i and k Sera
Re Examination of Factor e m and n Sera

By

GUNNAR HAUKENES

Received 28 viii 63

The i antigen was characterized by *Oeding* (1953a) as relatively heat stable and trypsin sensitive and presumed to be a protein Like the
ften found to be blocked in live
by the use of autoclaved organ
nutrient agar cultures was found
to be greater in 24 hours cultures than in 5 hours cultures in contrast to most other agglutinogens (*Oeding* 1957) *Grun* (1959), who used a modified slide agglutination technique arrived at the same conclusion with regard to the optimal age of the culture

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Cohen & Oeding (1962) compared the results of serological typing of pyogenic staphylococci in *i* and *k* factor sera by the agglutination and fluorescent antibody staining methods and obtained good agreement However while both strains F 21 and Wood 46 agglutinated in the *i* serum only strain F 21 reacted by the fluorescent antibody technique

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The present article deals with the antibody composition of the *i* and *k* factor sera In addition the *e m*, and *n* factor sera have been re examined with the complete set of type strains

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SEROLOGICAL TYPING OF *STAPHYLOCOCCUS AUREUS*

5 *Factor* ι *and* k *Sera*
Re Examination of Factor e, m, *and* n *Sera*

By

GUNNAR HAUKENES

Received 28 viii 63

The ι antigen was characterized by *Oeding* (1953a) as relatively heat stable and trypsin sensitive and presumed to be a protein Like the *h* antigen, the ι antigen was very often found to be blocked in live nutrient agar cultures, but unveiled by the use of autoclaved organisms Later the agglutinability of live nutrient agar cultures was found to be greater in 24 hours cultures than in 5 hours cultures in contrast to most other agglutinogens (*Oeding* 1957) *Grun* (1959), who used a modified slide agglutination technique, arrived at the same conclusion with regard to the optimal age of the culture

When pyogenic staphylococci were typed serologically, it was commonly experienced that strains which had the *e* antigen also agglutinated *in ι and k sera The cause of this cross-agglutination* appeared to be the *m* antigen as discussed by *Haukenes & Oeding* (1960) As a consequence the ι and *k* sera have now been prepared by an additional absorption with strain 1503 to remove *m* agglutinins

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The present article deals with the antibody composition of the ι and *k* factor sera In addition the *e*, *m*, and *n* factor sera have been re-examined with the complete set of type strains

MATERIALS AND METHODS

The immunization, agglutinin absorption and agglutination procedures are the same as used in earlier studies (*Oeding* 1957, *Haukenes & Oeding* 1960 *Haukenes* 1963 a)

EXPERIMENTAL AND RESULTS

Factor i Serum

The i factor serum is prepared by absorption of serum F 21 with strains 2095 and 1503 With our present knowledge of the antigens of strain F 21 the following antibodies might be found in an F 21 immune serum : b_1, b_2, b_3, ac_1, c_1 (4) and m (6) in addition to the i antibodies Strain 2095 removes b_1, b_2, b_3 and ac_1 antibodies, and strain 1503 removes m antibodies (6) Thus possible c_1 and i antibodies will be left Our F 21 immune serum possessed strong b_1, m, and i antibodies, but no detectable c_1 antibodies when tested with the reference strains 1015 and 6376 However, to be sure that all c_1 antibodies are removed the i serum studied here has been prepared by absorption with strain 3647 instead of strain 2095 in addition to strain 1503

TABLE 1

Agglutinability of the Type Strains in i Factor Serum and Agglutination Patterns after Re absorption of the i Factor Serum with Type Strains

Strain	i factor serum	i factor serum absorbed with			
		1503	365	F 21	Wood 46
F 21	+++(500)	+++	+++	—	++
Wood 46	+++(100)	+++	+	—	—
Other type strains	—				

Code +, ++, +++ Strength of agglutination
— No agglutination
Figures in brackets denote reciprocals of agglutination titres
Factor i serum was prepared by absorption of serum F 21 with strains 1503 and 3647

The agglutinability of the type strains in an i factor serum has been shown in Table 1 together with the agglutination patterns obtained after re-absorption with the type strains It appears that only strains F 21 and Wood 46 were agglutinated In contrast to *Oeding*'s findings (10) strain Wood 46 agglutinated considerably stronger with 5 hours cultures than with 24 hours The re-absorption experiments indicate the presence of at least two different antibodies which are designated i_1 and i_2 Strains F 21 and Wood 46 possess the i_1 agglutinogen and yield the i_1 line on agar precipitation (11) In agreement with earlier investigations (6) strain 365 also absorbed i_1 agglutinins (and precipitins) although weakly, but no i_1 antigen could be demonstrated in strain Cowan III No i_1 agglutination or i_1 line could be demonstrated after autoclaving of the organisms

After re-absorption of the i serum with strain Wood 46 only strain F 21 agglutinated Repeated absorptions with strain Wood 46 were performed to ensure that all i_1 agglutinins has been removed, but agglutinins were still left to strain F 21 Agar precipitation experiments confirmed that all i_1 antibodies had been removed It was of considerable interest to see whether these residual agglutinins, named i_2, could be absorbed by any of the other type strains, since F 21 immune serum is used for preparation of m factor serum as well as i Absorption experiments showed, however, that the other type strains failed to absorb i_2 agglutinins

The use of mannitol-salt agar cultures for agglutination gave weaker reactions than nutrient agar cultures After autoclaving several type strains agglutinated weakly, *i e* strains 2253, 28, F 21, Wood 46, Cowan I, Cowan II, and Cowan III, but in the re-absorbed sera the reactions were either lost or too weak for identification of the antibody

Preparation of i_1 and i_2 factor sera As mentioned above serum F 21 has to be absorbed with strains 1503 and 3647 for preparation of i_1 factor serum but none of the type strains other than strain F 21 were able to absorb i_2 antibodies Other strains were searched for in which the i_2 antigen was demonstrable, but they were all in possession also of the i_1 and m antigens A pure i_1 factor serum could therefore not be prepared from the F 21 immune serum used in the present investigations Other F 21 immune sera, however, have been found to contain *no* detectable i_2 antibodies, *e g* the serum used in (6) and the i_1 and m sera used by *Hofstad* (7)

Strain Wood 46 immune serum contained too weak i_1 antibodies for preparation of the factor serum

Factor i_2 serum can be prepared by absorption of serum F 21 with strains 1503, 3647, and Wood 46

Factor k Serum

This serum is prepared by absorption of serum 365 with strains 1503 and 2093 Strain 365 has been found to have the antigens a_2, b_1, b_2, e, h [illegible], m and n (4, 5, 6) in addition to the [illegible]

[illegible] performed

In the present investigations *serum 365* was first absorbed with strains 1503 and 3647 and thereafter with each of the other type strains Three (or 4) patterns of reaction were obtained (Table 2), indicating at least 2 (or 3) different *antibodies* After absorption with strains Cowan I and F 21 the only strain which agglutinated was 365, and this agglutinogen has been designated k_1 The agglutinogen shared by strains 365 and Cowan I is designated k_2

The k_1 antigen was found to be heat-stable in accordance with

Oeding's findings (8), and the agglutinability of mannitol-salt agar cultures and autoclaved nutrient agar cultures was much greater than with the ordinary live nutrient agar cultures

The agglutinability of strain 365 with k_2 antibodies has not been determined, since we have at present no strain which selectively absorbs k_1 antibodies. No pure k_2 serum has therefore been prepared, and the absorbing capacity of the other type strains with regard to k_2 antibodies has not been examined

The weak agglutinins to strain F 21 have provisionally been designated k_3, but most probably they are k_1 antibodies since they are removed by absorption with strain Wood 46

TABLE 2

Agglutinability of the Type Strains in a k Factor Serum, and Agglutination Patterns after Re-absorption with Type Strains

Strain	Culture	k factor serum				
		Not re-absorbed	Re-absorbed with strains			
			Cowan I	I 21	365	Cowan I and I 21
365	nutrient agar	++	+	+	—	+
365	mannitol agar	+++	+++	+++	—	+++
I 21	nutrient agar	+	(+)	—	—	—
I 21	mannitol agar	+	(+)	—	—	—
Cowan I	nutrient agar	++	—	+	—	—
Cowan I	mannitol agar	++	—	++	—	—
Other type strains		—				

Code See Table 1 The k factor serum was prepared by absorption of serum 365 with strains 2095 and 1503

Factor e Serum

It has previously been proposed to prepare *e* serum from serum 1503 by absorption with strains 3647 and F 21 (6) With this technique the resulting *e* serum commonly agglutinated strain Cowan I in addition to the strains possessing the *e* antigen (cf also Table 3 in (6)) Later it has been found that this is due to a new agglutinogen which will be described in the next article in connection with the antigens of the Cowan strains The *e* factor serum should therefore be prepared by absorption of serum 1503 with strains 3647 and Cowan I, and this method was used for preparation of the *e* serum employed here

None of the new type strains, 670, 1015, 830, 5687, and 6376, were agglutinated by the *e* serum, and none of them absorbed *e* agglutinins Contrary to earlier findings (6) strain 365 was found to be unable to exhaust the *e* serum by absorption

It has regularly been difficult to prepare potent *e* factor serum, and it has hitherto been of little practical value in serological typing of staphylococci

Factor m Serum

This serum is prepared by absorption of immune serum F 21 with strains Wood 46 and Cowan III (6). The new type strains 670, 1015, 830, 5687 and 6376 were tested in the *m* serum and none of them were agglutinated. Strains 670 and 5687 were found to absorb *m* agglutinins but the absorbing capacity of the latter strain was weak and irregular. On re-examination of the other type strains and comparison with formerly obtained results (6) one striking difference was observed as our strain 28 seemed to have lost its *m* antigen. Agglutination with mannitol salt agar cultures was also attempted but with negative result. We have at present none of the original cultures of this strain since the strains were not stored freeze dried at that time. The formerly obtained agglutination with strain 28 was undoubtedly caused by the *m* agglutinin since examination of an earlier produced strain 28 immune serum revealed a content of *m* agglutinins. Absorption of *m* factor serum with strain Cowan II resulted in a reduction of the agglutination titres of the strains possessing the *m* agglutinogen.

The results reported above were also obtained with an *m* factor serum prepared from serum 1503.

Before using a F 21 immune serum for preparation of *m* factor serum it should be tested for the presence of ι antibodies, the presence of which will render it unsuitable for an *m* factor serum (cf. above).

It is commonly experienced that it is necessary to use the growth of 2 or 3 Roux bottles of strain Wood 46 bacteria for each 3 ml of diluted serum on preparation of *m* factor serum, while the standard dose of organisms is one Roux bottle. This is due to a more scanty growth of strain Wood 46 on nutrient agar than obtained with most other pyogenic staphylococci.

The agglutinability and absorbing capacity of strain Cowan III have appeared to be irregular, and it is recommended to replace strain Cowan III with strain 3647 when serum F 21 is absorbed for preparation of *m* factor serum. This modification will also make the procedures more uniform, since absorption with strain 3647 is widely used for preparation of factor sera.

Factor n Serum

This serum is prepared by absorption of serum Cowan III with strains [illegible] and F 21 (6). Originally absorption with strain F 21 was carried out because strain Cowan III was presumed to possess the ι antigen. As reported above strain Cowan III has no ι antigen, but with our present knowledge of the antigens of strain Cowan III absorption with strain F 21 is necessary to ensure removal of possible c_1 antibodies.

As pointed out in a previous paper (6) immune serum Cowan III has rather weak *n* antibodies as compared with serum 1503, but owing to lack of suitable strains for absorption, serum Cowan III was resorted to

Recently *Hofstad* (7) has found a strain which possesses the *e*, but not the *n* antigen, and this strain (strain 137) will make it possible to prepare an *n* factor serum from serum 1503

None of the new type strains, 670, 1015, 830, 5687, and 6376, were agglutinated by an *n* factor serum, and none of these absorbed *n* agglutinins The other type strains were re-examined with regard to their agglutinability in the *n* serum and ability to absorb *n* antibodies The agglutinability of strain Cowan III was found to be weaker and more irregular than before, and strain 365 was not able to absorb *n* antibodies

Distribution of the e, i_1, i_2, k_1, k_2, m, *and* n *Antigens Among the Type Strains (See Table 3)*

At present no complete list of strains possessing the k_2 antigen can be given since a pure factor serum has not been prepared

TABLE 3

Distribution of the e i_1 i_2 k_1 k_2 m *and* n *Antigens Among the Type Strains*

Strain	Antigens						
1503	*e*					*m*	*n*
2253	*e*					*m*	*n*
28	*e*					(m?)	*n*
365		i_1		k_1	k_2	*m*	
3647							*n*
I 21		i_1	i_2			*m*	
Wood 46		i_1					
Cowan I					k_2	*m*	
Cowan II						(m?)	
Cowan III							*n*
670						(*m*)	
5687						(*m*)	

Brackets denote that the antigen could only be demonstrated by absorption
The complete distribution of the k_2 antigen has not been examined

Preparation of Individual Factor Sera

Factor *e* serum	Absorption of serum 1503 with strains 3647 and Cowan I
Factor i_1 serum	Absorption of serum F 21 (which is controlled to have no i_2 antibodies) with strains 1503 and 3647
Factor i_2 serum	Absorption of serum F 21 (possessing i_2 antibodies) with strains 1503 3647, and Wood 46
Factor k_1 serum	Absorption of serum 365 with strains F 21 and Cowan I
Factor k_2 serum	Cannot be prepared at present A k_1k_2 serum is prepared by absorption of serum 365 with strain F 21

Factor *m* serum	Absorption of serum F 21 (controlled to have no 12 antibodies) with strains 3647 and *Wood 46*
Factor *n* serum	Absorption of serum Cowan III with strains 2095 and F 21 (Cf also *Hofstad* (7))

DISCUSSION

Oeding (1953b) and *Grun* (1959) found that the ι antigen occurred rather commonly among pyogenic staphylococci Their ι serum was prepared by absorption of serum F 21 with strain 2095 and it is probable that the serum has contained *m* antibodies However *Oeding* commonly demonstrated the ι antigen by the use of autoclaved organisms which do not agglutinate in an *m* factor serum Antibodies to the heat stable ci antigen might have been present but they cannot explain the agglutinations obtained with autoclaved organisms of strains 1503 28 2095 and 3189 Also in the present investigations weak reactions were obtained with autoclaved bacteria of some strains It is reasonable to presume that the agglutinins against autoclaved organisms are not identical to the ι agglutinins or other hitherto known agglutinins

of the respective factor sera in routine serological typing has not yet been settled

Cohen & Oeding (1) found good agreement between the agglutination and the fluorescent antibody methods with both ι and *k* factor sera They report however that only strain F 21 (and not strain Wood 46) was stained by the conjugated ι antibody reagent A possible explanation to this seems to be that their ι factor serum has contained ι antibodies

discrepancy in connection with the h_1 and *h* antigens

Oeding (10) and *Grun* (2) stress the importance of using 24 hours cultures instead of the ordinary 5 hours cultures when testing for the ι agglutinogen This has not been the author's experience and in the present investigations strain Wood 46 gave a considerably weaker reaction after 24 hours cultivation It is uncertain however whether the observations by the above authors apply to the ι antigens or the *m* antigen

In accordance with *Oeding's* findings (8) the h_1 antigen was found to be heat stable Like most other heat stable antigens it was partly blocked in live nutrient agar cultures and unmasked when mannitol salt agar cultures were used for agglutination

Re examination of factor *e m* and *n* sera gave results which differed a little from those previously obtained Further studies on the mechan

isms of antigen blocking may possibly provide more information of the cause of these discrepancies Otherwise, it has regularly been our experience that the antigenic make-up of a *Staphylococcus aureus* strain is a very stable and characteristic feature

The *m* antigen was found to be widely distributed among the type strains, and *Hofstad* (7) has found that most strains belonging to phage group I have the *m* antigen The antigen, however, was not specific to this phage group as was the 263-1 antigen described by the same author Forty-five strains belonging to phage group I were also tested in an *n* factor serum, but none of the strains were agglutinated No experience has been obtained with the *e* serum because agglutinins were too weak

SUMMARY

The antibody composition of the *i*, *k*, *e*, *m*, and *n* factor sera has been studied Two new antigens, i_2 and k_2, have been detected, but they seem to be of minor importance for serological typing The *e*, *m*, and *n* factor sera have been re-examined with the complete set of type strains

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TUBERCULIN PRODUCTION

6 *Relationship between pH of Culture Filtrate and Yield of Tuberculoprotein and Tuberculin*

By

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During growth of *Mycobacterium tuberculosis* on liquid medium, the pH of the culture filtrate varies, dependent on the strain and the composition of the medium (*Smith* 1905, 1910, *Griffith* 1907, *Lind* 1948, *Asami et al* 1953 a, b, *Sawada et al* 1955, *Svenkerud* 1955, *Takehara* 1957, *Asami et al* 1959 a, *Magnusson, Kim & Bentzon* 1963 a, b) In some previous studies, culture filtrates of *M tuberculosis* with low pH have shown smaller tuberculin yield and/or tuberculoprotein content than alkaline filtrates (*Wong* 1937, *McIntosh & Konst* 1949, *Corper & Cohn* 1950, *Paterson et al* 1958, *Magnusson & Bentzon* 1958, *Asami et al* 1959 a, b, c, 1961, *Magnusson, Kim & Bentzon* 1963 a, b) In contrast when comparing cultures of different ages, *Asami et al* 1952 and *Sawada et al* 1955 found the highest activity in the old filtrates with a fairly low pH In other studies (*Corper, Cohn & Bower* 1940, *Svenkerud* 1955, *Takehara* 1957, *Study Committe* 1959) no clear relationship could be found *Lind* (1948) and *Magnusson & Bentzon* (1958) observed that the relationship between the pH of the culture filtrate and the tuberculin yield varies for different strains

In the majority of these previous studies, the variations in pH are due to the use of different strains or media, differences in age or growth rate of the cultures, or to other factors In a few studies only the pH of the culture filtrate is varied deliberately after growth At the end of four weeks of growth, *Wong* (1937) adjusted the pH from 5 8 to 7 2 by means of sodium carbonate solution in one set of flasks of a culture of *M tuberculosis* on synthetic medium After thorough mixing, the flasks were incubated for two more weeks at 37°C together with control flasks The protein content in the alkaline filtrate was more than double that in the control flasks *McIntosh & Konst* (1943) found that the yield of PPD prepared from *M paratuberculosis* (Johnes bacillus)

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increased five-fold when the pH of the medium was changed from an acid (pH 5 5) to an alkaline reaction (pH 8 0) previous to harvesting The bottles were placed in the refrigerator for eight or ten days and agitated frequently before being sterilized by heating

Thus, except for these two latter studies, the variations in pH found in the previous works have been linked with other differences within the culture filtrates, and it is not known what influence this has had on the relationship under study

The purpose of the present experiment is to study the tuberculin activity and tuberculoprotein content of heat-sterilized culture filtrates of *M tuberculosis* in which the pH is changed immediately before sterilization by the addition of hydrochloric acid or sodium hydroxide

MATERIALS AND METHODS

Cultures A virulent strain of *M tuberculosis* F5, was cultivated at 38° C for six weeks in small flasks containing 180 ml *Sauton* medium (study I) In study II another strain of *M tuberculosis* E9656, was cultured for three or six weeks on *Lind bII* medium (*Lind* 1948) The method of preparing the two media was as described previously (*Magnusson, Kim & Bentzon* 1963a)

Before sterilization of the cultures by heating in streaming steam for one hour the pH was changed in each flask by the addition of hydrochloric acid or sodium hydroxide, except for one or two flasks in which the pH was not changed After sterilization, the dry weight of organism pH and protein content of the culture filtrate in each flask were measured as described previously (*Magnusson Kim & Bentzon* 1963a), except for the filtrate in study II to which 10 ml 5 N NaOH had been added In this case addition of the usual amount of 50 per cent trichloroacetic acid (1 1 ml) did not precipitate all the protein and a larger amount (4 4 ml) was therefore added

Tuberculin activity of culture filtrates The tuberculin activity of the culture filtrates was compared with that of standard dilutions containing 100 tuberculin units (TU) and 10 TU per 0 1 ml by intradermal testing on BCG vaccinated guinea pigs Dilutions of purified tuberculin (PPD) RT 22 Statens Seruminstitut, containing 0 0017 mg (100 TU) and 0 00017 mg (10 TU) per 0 1 ml were used as standard dilutions

Before injection the filtrates were diluted 1 20 1 100, 1 200 or 1 400 using phosphate buffered saline pH 7 38 as diluent Fresh dilutions were prepared on each testing day

Experimental animals Thirty two albino guinea pigs were used in study I and 64 in study II, all the animals were bred at the farm attached to Statens Seruminstitut and weighed 470–570 g Each animal was vaccinated intracutaneously in four sites on the abdomen with 0 1 ml of Danish BCG vaccine[1] ten times stronger than the standard vaccine Each animal thus received 3 mg (semi dried weight) BCG The vaccine batches were Nos 1150 1151 1154 and 1155 The animals were used four weeks after vaccination

Experimental design Twelve preparations divided into two groups (A and B) of 6 preparations were tested in study I The 24 preparations in study II were divided into four groups (A–D) with 6 in each The experiments were carried out on two days for each group the six preparations being injected together with the two standard dilutions into 8 sites on each of 8 animals according to a 8 × 8 latin square design Thus every test preparation was used for 16 injections

Tuberculin injection and reading The technique for injection and reading of the reactions was the same as described previously (*Kim Magnusson & Bentzon* 1963) The volume injected was 0 1 ml

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1 The authors are grateful to Dr *A Tolderlund* and Miss *K Bunch Christensen* BCG Department, Statens Seruminstitut for preparing and supplying the vaccine

TABLE 1

Bacterial Crop (in mg per Flask), Tuberculoprotein Content (in µg per ml), Mean Size of Intradermal Reactions (Erythema in mm) of BCG Vaccinated Guinea Pigs Read after 24 and 48 Hours and Tuberculin Activity (in TU per ml) of Heat Sterilized Culture Filtrate from 6 Week Old Cultures of M. tuberculosis strain H5 on Sauton Medium in Relation to pH of the Filtrate varied by addition of Hydrochloric acid or Sodium hydroxide before Sterilization

Acid for pH adjustment (ml)	pH of culture filtrate	Dry weight of organism* (mg)	Tuberculoprotein (µg/ml)	Study	Dilution	Tuberculin activity: 24 hours: Mean reaction (mm)	24 hours: Activity§ (TU/ml)	48 hours: Mean reaction (mm)	48 hours: Activity† (TU/ml)
0.5 N HCl 7.5	3.3	1000	0.09	I A	1:100	15.3	67 000	12.0	65 000
0.5 N HCl 5.0	3.8	1100	0.01	I A	1:100	14.1	44 000	11.2	47 000
0.5 N HCl 3.0	4.2	1150	<0.01	I A	1:100	13.3	34 000	10.7	39 000
0.5 N HCl 3.0	4.3	1200	<0.01	I A	1:100	13.1	32 000	9.7	26 000
0.5 N HCl 1.05	4.9	1250	0.01	I A	1:100	13.7	33 000	10.9	42 000
none	5.4	1200	0.12	I A	1:100	13.7	39 000	10.1	30 000
0.5 N NaOH 1.5	5.9	1100	0.13	I B	1:400	11.8	72 000	9.0	72 000
0.5 N NaOH 2.5	6.5	1200	0.44	I B	1:400	12.4	88 000	9.6	89 000
0.5 N NaOH 3.5	7.4	1100	0.77	I B	1:400	14.0	155 000	10.5	125 000
0.5 N NaOH 5.5	8.2	1050	1.03	I B	1:400	13.7	140 000	10.3	115 000
0.5 N NaOH [illegible]	8.6	900	1.53	I B	1:400	14.4	175 000	10.8	140 000
0.5 N NaOH 13.0	8.7	900	1.65	I B	1:400	13.5	130 000	10.5	125 000

	24 hours	48 hours
Mean reaction to standard dilution in Study I A 100 TU	16.5 mm	13.5 mm
Mean reaction to standard dilution in Study I A 10 TU	9.7 mm	7.3 mm
Mean reaction to standard dilution in Study I B 100 TU	16.8 mm	13.7 mm
Mean reaction to standard dilution in Study I B 10 TU	10.1 mm	7.4 mm

Each mean is based on 16 reactions
* 180 ml medium
§ 95 per cent confidence limits 78 and 128 per cent
† 95 per cent confidence limits 76 and 131 per cent

increased five-fold when the pH of the medium was changed from an acid (pH 5 5) to an alkaline reaction (pH 8 0) previous to harvesting The bottles were placed in the refrigerator for eight or ten days and agitated frequently before being sterilized by heating

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MATERIALS AND METHODS

Cultures A virulent strain of *M tuberculosis* F5, was cultivated at 38° C for six weeks in small flasks containing 180 ml *Sauton* medium (study I) In study II another strain of *M tuberculosis* E9656 was cultured for three or six weeks on *Lind bII* medium (*Lind* 1948) The method of preparing the two media was as described previously (*Magnusson Kim & Bentzon* 1963a)

Before sterilization of the cultures by heating in streaming steam for one hour the pH was changed in each flask by the addition of hydrochloric acid or sodium hydroxide, except for one or two flasks in which the pH was not changed After sterilization, the dry weight of organism pH and protein content of the culture filtrate in each flask were measured as described previously (*Magnusson Kim & Bentzon* 1963a), except for the filtrate in study II to which 10 ml 5 N NaOH had been added In this case addition of the usual amount of 50 per cent trichloroacetic acid (1 1 ml) did not precipitate all the protein and a larger amount (4 4 ml) was therefore added

Tuberculin activity of culture filtrates The tuberculin activity of the culture filtrates was compared with that of standard dilutions containing 100 tuberculin units (TU) and 10 TU per 0 1 ml by intradermal testing on BCG vaccinated guinea pigs Dilutions of purified tuberculin (PPD) RT 22 Statens Seruminstitut, containing 0 0017 mg (100 TU) and 0 00017 mg (10 TU) per 0 1 ml were used as standard dilutions

Before injection the filtrates were diluted 1 20 1 100 1 200 or 1 400 using phosphate buffered saline pH 7 38 as diluent Fresh dilutions were prepared on each testing day

Experimental animals Thirty two albino guinea pigs were used in study I and 64 in study II, all the animals were bred at the farm attached to Statens Seruminstitut and weighed 470–570 g Each animal was vaccinated intracutaneously in four sites on the abdomen with 0 1 ml of Danish BCG vaccine[1] ten times stronger than the standard vaccine Each animal thus received 4 mg (semi dried weight) BCG The vaccine batches were Nos 1150 1151 1154 and 1155 The animals were used four weeks after vaccination

Experimental design Twelve preparations divided into two groups (A and B) of 6 preparations were tested in study I The 24 preparations in study II were divided into four groups (A D) with 6 in each The experiments were carried out on two days for each group the six preparations being injected together with the two standard dilutions into 8 sites on each of 8 animals according to a 8 × 8 latin square design Thus every test preparation was used for 16 injections

Tuberculin injection and reading The technique for injection and reading of the reactions was the same as described previously (*Kim Magnusson & Bentzon* 1963) The volume injected was 0 1 ml

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1 The authors are grateful to Dr *K Tolderlund* and Miss *K Bunch Christensen* BCG Department, Statens Seruminstitut for preparing and supplying the vaccine

TABLE 2

l u t (g l k Tal t (s l 7 l r l M S z of l al l Re t s (l y ( ) f
l (l l l os l l f r 24 48 ll d bll l l u l ll l l ll S z (l l l
fr n 7 B k ll (ul s f M tub s s s F l r Sol l lr x l b fo e S l za R l o o l ll of l l ll ate V a l by Sd l o f
Hy l ll

A a g	of u n	ry w g of o g g	lu o g	S l	l	Tu u a — 24 hours: a n o	A l L §	48 l o rs: Mea n	A n † l L
4 N HCl 4	2 2	550	0 07	II A	1 200	14 4	30 00	12 3	42 000
5 N HCl 2	3 4	750	0 0	II B	1 200	14 7	32 000	12 5	35 0 0
4 N l (l 2	3 J	7	0 004	II A	1 200	13 3	16 0	11 3	25 0 0
0 5 N HCl 15	4 2	700	0 0 J	II B	1 200	13 3	15 000	10 8	15 000
0 5 N HCl 10	5 7	750	0 0(	II A	1 2 0	13 1	14 00	1 9	20 000
	7 3	9 0	0 4	II B	1 200	15 8	J8 00	13 J	53 000
	8 1	750	0 25	II A	1 200	14 J	40 000	12 8	54 000
0 5 N NaOH 10	8 8	800	0 23	II B	1 200	14 5	2J 00	12 0	27 0
5 N NaOH 15	J 1	1000	0 40	II A	1 20	1J 1	4J 0 0	12 9	J7 000
0 5 N NaOH 2	J 3	10 0	0 5J	II B	1 200	15 5	50 000	13 2	50 000
J N NaOH J	J (	75	0 21	II A	1 20	16 6	11 000	14 4	12 000
5 N NaOH 5	10.5	450	0 20	II B	1 20	13 3	1 50	11 2	1 800

	24 l ur	48 l o r
M an rea t to an lard dil ti in Study II A 1 0 Tl	17 7 m	15 3 m
M a r a ti to s an lard l luti in Study II A 10 Tl	13 7 m n	10 J n
Mea rea ti n to andard l luti in Study II B 100 Tl	18 1 m	15 J n
M an rea t n to sta lard dilut in St dy II B 10 Tl	13 8 m n	11 4 mn

l a h m an i ba d on 16 react o s
180 l m l un
§ 95 l r ce t c f d n l i stu ly II A 71 an l 141 l r c t s udy II B 73 an l 137 l er t
† 95 l er cent co fid nce l m t stu ly II A 70 and 143 l er cent stu ly II B 70 a l 142 l er c nt

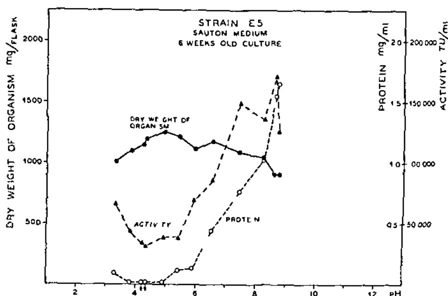


Fig 1

Dry weight of organism (●), tuberculoprotein content (○) and tuberculin activity (▲) of heat sterilized culture filtrate from 6 week old cultures of *Mycobacterium tuberculosis* strain E5 cultured on Sauton medium in relation to pH of the filtrate varied by addition of hydrochloric acid or sodium hydroxide before sterilization

Calculation of tuberculin activity The tuberculin activity D—in TU per ml of culture filtrate—was calculated from the mean size of reactions by the method reported previously (*Magnusson Kim & Bentzon* 1963b) The 95 per cent confidence limits for the activity are $D \times 10^{+-SE^*}$

RESULTS

Dry weight of organism The bacterial crop at pH 4 0 6 5 for E5 cultured on Sauton medium was approximately 1200 mg per flask and slightly lower at extreme values of pH (Table 1, Fig 1)

For E9656 cultured on Lind bII medium the total crop of bacteria was smaller in 3-week-old cultures, *viz* about 750 mg per flask (Table 2 and Fig 2), than in 6-week-old cultures, *viz* about 1100 mg per flask (Table 3 and Fig 3) There were large differences in crop between flasks (Figs 2 and 3) At pH > 12 the bacteria seemed to be destroyed (Fig 3)

Protein yield The change in the pH of the culture filtrates had a marked influence on the protein content (Tables 1 3 and Figs 1 3) The protein content was minimal (< 0 03 mg ml) at about pH 4 and maximal (0 55–1 65 mg ml) at about pH 9 (Tables 1 3) Adjustment of pH to values beyond the 4 9 range was carried out for the cultures

* Based on the average results for the two readers the following estimates of the standard deviation (s) were found Study I 1 18 (24 hours) 1 19 (48 hours) Study II 0 97 (24 hours), 1 11 (48 hours)

The standard error is calculated as $SE = \frac{s}{b_{ST}} \times \sqrt{\frac{15}{16}}$ where b_{ST} = average slope of the tuberculin dose response curve

TABLE 2

Bacterial Crop (in mg per Flask), Tuberculoprotein Content (in mg per ml), Mean Size of Intradermal Reactions (Erythema in mm) of BCG Vaccinated Guinea Pigs Read after 24 and 48 hours and Tuberculin Activity (in TU per ml) of Heat Sterilized Culture Filtrate from 3 Week old Cultures of M. tuberculosis strain PN 56 on I and bII Medium in Relation to pH of the Filtrate Varied by Addition of Hydrochloric acid or Sodium hydroxide before Sterilization

Addition for pH adjustment: Agent (ml)	pH of culture filtrate	Dry weight of organism* (mg)	Tuberculoprotein (mg/ml)	Study	Dilution	Tuberculin activity: 24 hours: Mean reaction (mm)	Tuberculin activity: 24 hours: Activity§ (TU/ml)	Tuberculin activity: 48 hours: Mean reaction (mm)	Tuberculin activity: 48 hours: Activity† (TU/ml)
4 N HCl 4	2.2	550	0.07	II-A	1:200	14.4	30,000	12.3	42,000
0.5 N HCl 20	3.4	750	0.05	II-B	1:200	14.7	32,000	12.5	35,000
4 N HCl 2	3.9	750	0.004	II-A	1:200	13.3	16,000	11.3	25,000
0.5 N HCl 15	4.2	700	0.003	II-B	1:200	13.3	15,000	10.8	15,000
0.5 N HCl 10	5.7	750	0.06	II-A	1:200	13.1	14,000	10.9	20,000
none	7.3	950	0.45	II-B	1:200	15.8	58,000	11.5	59,000
none	8.1	750	0.25	II-A	1:200	14.9	40,000	12.8	54,000
0.5 N NaOH 10	8.8	800	0.23	II-B	1:200	14.5	29,000	12.0	27,000
0.5 N NaOH 15	9.1	1000	0.40	II-A	1:200	15.1	45,000	12.9	57,000
0.5 N NaOH 20	9.3	1050	0.55	II-B	1:200	15.5	50,000	13.2	50,000
5 N NaOH 3	9.6	750	0.21	II-A	1:20	16.6	11,000	14.4	12,000
5 N NaOH 5	10.5	450	0.20	II-B	1:20	13.3	1,500	11.2	1,800

	24 hours	48 hours
Mean reaction to standard dilution in Study II-A 100 TU	17.7 mm	15.3 mm
Mean reaction to standard dilution in Study II-A 10 TU	13.7 mm	10.9 mm
Mean reaction to standard dilution in Study II-B 100 TU	18.1 mm	15.9 mm
Mean reaction to standard dilution in Study II-B 10 TU	13.8 mm	11.4 mm

Each mean is based on 16 reactions

* 180 ml medium

§ 95 per cent confidence limits study II-A 71 and 141 per cent, study II-B, 73 and 137 per cent

† 95 per cent confidence limits study II-A 70 and 143 per cent, study II-B 70 and 142 per cent

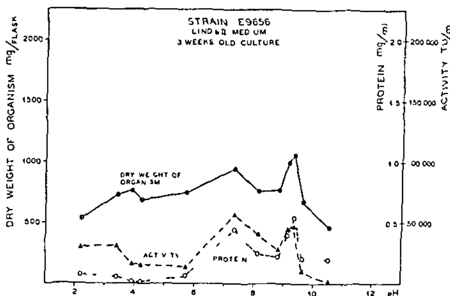


Fig 2

varied by addition of hydrochloric acid or sodium hydroxide before sterilization of strain E9656 Relatively higher protein yields were found at pH < 4, whereas an increase of pH above 9 caused a decrease in tuberculoprotein content (Figs 2 and 3)

The protein content of the 6-week old cultures of E9656 (Fig 3) was larger than that of the 3-week-old cultures (Fig 2)

Tuberculin activity The tuberculin activity was minimal—14,000-32,000 TU/ml—at pH 3 4 5 7, and maximal—58,000-175,00 TU/ml—at pH 7 3 8 6 estimated on the basis of the readings after 24 hours (Tables 1 3) The curves for the activity seem to follow those for the protein yield (Figs 1-3) However, the tuberculin activity per mg of protein depends on the pH (Fig 4) The relative activity is maximal at about pH 4—more than 2,000,000 TU/mg—and decreases for pH > 5 the higher the pH, independent of the age of the cultures At pH 8 5 the relative activity is approximately 120,000 TU/mg

The activities calculated on the basis of observations after 48 hours are very much the same as those based on the 24-hour-readings (Tables 1-3), except for studies II A and C (Tables 2 and 3) There the activities calculated on the basis of the observations after 48 hours were slightly higher than after 24 hours

DISCUSSION

A relationship between the *tuberculin activity* of heat-sterilized culture filtrates of *M tuberculosis* and the pH of the filtrates at the time of sterilization could be found in the present study The activity was mini-

TABLE 3

Bacterial Crop (in mg per Flask), Tuberculoprotein Content (in mg per ml), Mean Size of Intradermal Reactions (Erythema in mm) of BCG Vaccinated Guinea Pigs Read after 24 and 48 Hours, and Tuberculin Activity (in TU per ml) of Heat Sterilized Culture Filtrate from 6 Week Old Cultures of M. tuberculosis strain EJC56 on Long BH Medium in Relation to pH of the Filtrate Varied by Addition of Hydrochloric acid or Sodium hydroxide before Sterilization

Addition for pH adjustment: Agent	(ml)	pH of culture filtrate	Dry weight of organisms* (mg)	Tuberculoprotein (mg/ml)	Study	Dilution	Intracutaneous activity: 24 hours: Mean reaction (mm)	Activity § (TU/ml)	48 hours: Mean reaction (mm)	Activity † (TU/ml)
4 N HCl	4	1.8	650	0.48	II C	1:200	14.7	49 000	13.4	75 000
4 N HCl	2.5	2.5	750	0.50	II D	1:200	15.1	65 000	13.2	77 000
1 N HCl	7.5	3.4	900	0.15	II C	1:200	13.8	31 000	12.5	48 000
1 N HCl	5	4.0	1000	0.02	II D	1:200	14.2	42 000	12.1	45 000
1 N HCl	2.5	6.1	1050	0.21	II C	1:200	14.8	52 000	13.0	62 000
none		7.5	800	0.31	II D	1:200	16.3	115 000	13.7	93 000
none		8.3	1350	1.00	II C	1:200	16.5	120 000	14.4	125 000
1 N NaOH	2.5	8.7	850	0.99	II D	1:200	16.2	110 000	13.6	94 000
1 N NaOH	5	9.4	1100	0.86	II C	1:200	16.2	105 000	14.3	115 000
5 N NaOH	2	10.0	1100	1.01	II D	1:200	14.8	56 000	12.4	52 000
5 N NaOH	5	12.0	700	0.68	II C	1:20	13.1	2 200	11.2	2 600
5 N NaOH	10	12.3	250	0.58	II D	1:20	12.0	1,400	9.9	1 500

	24 hours	48 hours
Mean reaction to standard dilution in Study II C 100 TU	17.5 mm	15.4 mm
Mean reaction to standard dilution in Study II C 10 TU	12.9 mm	10.7 mm
Mean reaction to standard dilution in Study II D 100 TU	17.4 mm	15.1 mm
Mean reaction to standard dilution in Study II D 10 TU	12.7 mm	10.5 mm

Each mean is based on 16 reactions

* 180 ml medium

§ 95 per cent confidence limits study II C 74 and 135 per cent, study II D 75 and 134 per cent

† 95 per cent confidence limits study II C 71 and 140 per cent study II D 71 and 141 per cent

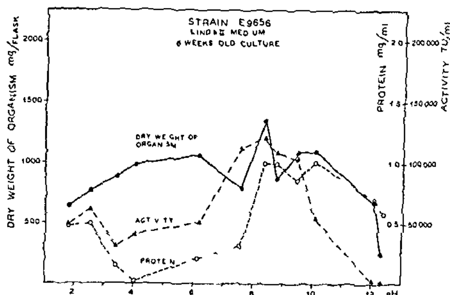


Fig 3

varied by addition of hydrochloric acid or sodium hydroxide before sterilization

mal at pH 3 4–5 7 and maximal at pH 7 3 8 6 Two previous works (*Asami et al* 1959 a, b) also indicate that there is a relationship between the tuberculin activity of culture filtrates and the pH

Both in a series of previous studies and in the present work, a relationship was also found between the *tuberculoprotein content* (*Wong* 1937) or the yield of purified tuberculin PPD (*McIntosh & Konst* 1949, *Paterson et al* 1958, *Magnusson & Bentzon* 1958) of heat-sterilized culture filtrates of *M tuberculosis* and the pH of the filtrates at the time of sterilization In the present study, the protein content seems to be maximal at about pH 9 and minimal at about pH 4 5, which is the isoelectric point of heated tuberculoproteins (*Long & Seibert* 1926, *Hanan & Ericks* 1937) *Lind* (1948) obtained only a minute yield of tuberculin from 5 to 20-week-old cultures of *M bovis* and *M avium* in two studies, *and assumed that the two strains had ceased to produce tuberculin*-active substance However, the pH of the filtrates was 4 8 5 5 and, on the basis of the results of the present study, this is more likely to be the explanation of the small yield

The relationship between protein content and pH seems to be slightly different from the relationship of tuberculin activity to pH in the present study There would appear to be stronger tuberculin activity per mg of protein at pH 4 5 than at pH 8 5, *i e* more than 2,000 000 TU as compared to 120,000 TU This is not found with PPD preparations (*Lind* 1948, *Asami et al* 1959 a), probably because part of the activity remains in the supernatant when acid culture filtrates are precipitated

In the present study, the tuberculin yield on Sauton medium seems

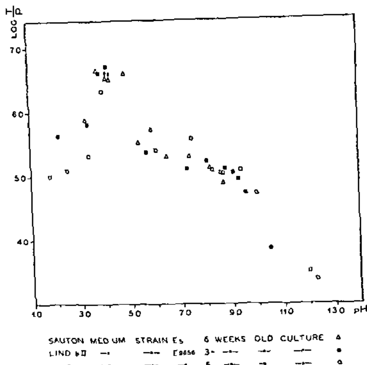


Fig 4

Tuberculin activity of tuberculoprotein in relation to pH of heat sterilized culture filtrate of *Mycobacterium tuberculosis* varied by addition of hydrochloric acid or sodium hydroxide before sterilization

Abscissa pH of culture filtrate
Ordinate Logarithm of tuberculin activity (in TU) per mg protein

to increase if the pH is changed from 5 4 to 7–9 by adding sodium hydroxide before heat sterilization of the cultures (Table 1) This has also been the experience in this laboratory[1], and in addition applies to production of sensitins from other mycobacteria (*McIntosh & Konst* 1943, *Magnusson* 1961, *Magnusson, Engbæk & Bentzon* 1961) The results of the present study also show that the tuberculin activity is destroyed if excessive amounts of sodium hydroxide are added (Tables 2 and 3) In the routine work in this laboratory, two out of 20 100 one-litre culture flasks *are sterilized and the pH measured, after which an* estimated amount of sodium hydroxide (usually 5 10 ml 1 N to one litre of medium) is added to the other culture flasks before heat sterilization In this way the risk of laboratory infection is avoided when measuring the pH in the non-sterilized cultures

Another solution to the problem of obtaining a high pH in the culture filtrate at the time of sterilization is to change the composition

[1] The studies have been performed at the Tuberculin Laboratory of Statens Seruminstitut Copenhagen

of the medium To that end, *Lind* (1948) increased the amount of potassium phosphate in Sauton medium (bII medium) and *Svenkerud* (1955) included sodium hydroxide and acetic acid in the same medium Larger yields of tuberculin were obtained by this means but the activity per mg of substance decreased (*Lind* 1948) In the present study, the reaction of Lind bII medium without adjustment was pH 7–8 both after 3 weeks and after 6 weeks, and no increase in the activity of the filtrates was seen as the result of the addition of sodium hydroxide (Tables 2 and 3)

Since different strains of *M tuberculosis*, and especially strains of different species of *Mycobacterium*, induce *different* changes in pH during growth, it is hardly possible to indicate the composition of any *one medium which will always have a high terminal pH independent* of the strain (*Svenkerud* 1955) On the other hand, from the point of view of the comparability of the preparations, it is preferable to use the *same* medium for preparation of sensitin from different strains The present procedure, *viz* adjustment of pH of acid culture filtrates to pH 7–9 before heat-sterilization of the cultures, would therefore be preferable, especially when sensitin (tuberculin) is to be prepared from many different strains

Shiraishi (1952) and *Ohtomo* (1954) found that the tuberculin activity of culture filtrate of *M tuberculosis* was not influenced by adjustment of pH when heat-sterilization was carried out *after* removal of the bacteria by filtration The probable reason for the larger yield of tuberculoprotein by adjusting the pH before heat-sterilization is, therefore, that larger amounts of protein are extracted from the cells at the higher pH than at the lower pH (*Heidelberger & Menzel* 1934, *Hanan & Zurett* 1936, *Menzel & Heidelberger* 1938, *Paterson* 1948, *McIntosh & Konst* 1949, *Ohtomo* 1955, *Hirai* 1958) and that part of the protein is precipitated if the pH is near the isoelectric point (*Paterson* 1948, *Corper & Cohn* 1950)

In two of the present studies (II A and II C), the tuberculin activities calculated on the basis of observation after 48 hours were systematically *higher* than after 24 hours The opposite was found in a previous study (*Kim, Magnusson & Bentzon* 1963), while in other studies in the present series no systematic differences were seen As mentioned previously (*Kim, Magnusson & Bentzon* 1963), use of one set of average values (bST and TRST) for the 24-hour comparison and another set for the 48 hour comparison may account for the systematic trend

SUMMARY

Prior to heat-sterilization of three to six-week old cultures of *Mycobacterium tuberculosis* grown on synthetic medium, the pH of the culture filtrates was changed by addition of hydrochloric acid or sodium hydroxide

The tuberculin activity of the culture filtrates, measured by intradermal tests on BCG vaccinated guinea pigs, was minimal at pH 3 4–5 7 and maximal at pH 7 3–8 6

The tuberculoprotein content of the filtrates seemed to be minimal at pH 4–5 and maximal at about pH 9

The activity per mg of tuberculoprotein was much higher at pH 4–5 than at pH 8 5

A change in the pH of acid culture filtrates to pH 7–9 prior to heat-sterilization is proposed in order to obtain an increased yield of tuberculin

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THE RELATION BETWEEN INOCULUM AND ZONE SIZE IN SENSITIVITY TESTS BY THE AGAR DIFFUSION METHOD

With a Special View to the Importance of Prediffusion

By

V FROLUND THOMSEN

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cent inhibitory concentration as determined by the plate dilution method The correlation was found to vary with different groups of bacteria *depending on their rate of growth, and it was shown that the pre*diffusion technique could reduce the variances arising from this The present communication describes some studies on the relationship of the inoculum and zone size, and the influence of prediffusion on this relationship

Most of the published studies on the significance of the inoculum have been performed with diffusion and growth commencing simultaneously It is generally agreed that under such conditions the inoculum is of decisive importance for the size of the inhibition zone, as increasing inoculum—other things being equal—results in decreasing diameter of the inhibition zone (19, 8, 6, 7 15, 13) The inoculum size is of particular importance in sensitivity tests of penicillinase producing staphylococci to penicillin as these organisms may show complete resistance *with a large inoculum an sensitivity with a small one* (17, 23, 1 8 2 21) Tests with sulfonamides show a similar feature, in that a large inoculum may result in an apparent resistance of many sensitive strains (10 12, 3, 11)

Attempts have been made, by various means, to eliminate the inoculum variations in the agar diffusion technique Primarily, an attempt was made to standardize the inoculum so as to give dense but not confluent colonies (19, 7) Such a procedure requires sub culturing and therefore cannot be used *when the sensitivity tests are performed* 'directly on the material *Jensen & Kiær* (14) suggested that instead of attempting uniform density of growth, the plate should be seeded with

These investigations were aided by a grant from *P Carl Petersens Fond*

decreasing density centrally, whereupon the result can be read at the suitable density The disadvantage of this method is that the inhibition zones are not circular, and that each single field must be seeded separately

A number of mutually independent mechanisms can be considered responsible for the inoculum effect in sensitivity tests, depending on the method chosen, as it is possible to distinguish between the inoculum effect in the dilution method and in the diffusion method *Cooper et al* (4) and *Linton* (16) have studied the relationship between the inhibition zone and the inoculum size, by means of a one-dimensional diffusion method They used streptomycin as antibiotic and a Klebsiella and a staphylococcal strain as organisms They found a simple relationship between the size of the inhibition zone and inoculum maintaining that the inoculum effect was based on the number of generations saved in obtaining a constant critical number when the inoculum is increased However, they paid no attention to the rôle of prediffusion

Another inoculum-induced variation is that of the penicillinase-forming staphylococci on penicillin where the variation is associated with the action of penicillinase If this enzyme is present in the inoculum its amounts are proportional to the bacterial count With a small inoculum the amount of penicillinase is small, and only a small amount of penicillin is split, so that even low initial concentrations of penicillin are growth-inhibiting With a large noculum the amount of pencillinase transferred to the substrate is large, and much penicillin is destroyed, so that even high initial concentrations have no effect This inoculum-induced variation will be seen both in tests by dilution methods and by diffusion methods, and is difficult to distinguish from "true" penicillin-resistance of the staphylococci A similar inoculum effect is known in the case of the sulfonamides, although the mechanism is different, depending on competition between sulfonamide and antagonists in the inoculum When the inoculum is increased, the total amount of antagonists increases simultaneously, resulting in a retarded effect of the sulfonamide The visible result is smaller inhibition zones, and with a very large inoculum no inhibition zone will appear This effect is seen both with the dilution mehtod and with the diffusion method

In tube dilution method the presence of a few bacteria of a resistant mutant may give turbidity and with increasing inocula the probability of transferring more resistant variants also increases Using solid media the resistant variants will give rise to only a few colonies, and if the method of *Karber* (9) is used to calculate the 50 per cent inhibitory concentration, the inoculum effect is avoided in principle, as inoculating an increasing number of bacteria gives not only an increased number of resistant variants but also a corresponding increase in the number of sensitive organisms, so that the ratio between resistant and sensitive organisms remains constant

The purpose of the present study has been to determine the signific-

ance of prediffusion for the inoculum effect, using the agar diffusion method As a measure of the effect, an inoculum index was calculated as follows

$$I = \frac{\text{inhibition zone (1 1000)} \quad \text{inhibition zone (1 1)}}{\text{inhibition zone (1 1)}} \times 100$$

where I = inoculum index in per cent, inhibition zone (1 1000) — mean diameter of inhibition zones with inoculum diluted 1 1000, inhibition zone (1 1) = mean diameter of inhibition zones with undiluted 1 1 inoculum The calculation is possible only when the strain gives measurable zones of inhibition with undiluted culture This index which is easy to compute is positive and increases with increasing inoculum-dependency

MATERIAL AND METHODS

Prep
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TABLE 1

Concentration of Antibiotic Solutions and Disc Content

Antibiotic	Solution	Disc strength
Penicillin	150 units/ml	4 units disc
Penicillin (mega units)	9400 units/ml	250 units/disc
Sulphathiazole	7550 µg/ml	200 µg/disc
Streptomycin	1900 µg/ml	50 µg/disc
Tetracyclin	3800 µg/ml	100 µg/disc
Chloramphenicol	1900 µg ml	50 µg/disc
Erythromycin	375 µg/ml	10 µg/disc

For practical reasons an inactive dye has been added to the antibiotic solutions

before each experiment

Inoculation Each plate is seeded in the following manner 0 1 ml of the suspension is delivered by means of a sterile pipette and then distributed uniformly over the whole plate with the aid of a sterile bent glass rod The plates are immediately placed in an incubator ot 36 3° C and left overnight the inhibition zones thus produced are then measured

Determination of size of inoculum The 1 1000 diluted inoculum is further diluted 1 1000 and 0 1 ml of this dilution is spread on a plate After incubation overnight

periments the medium was used which is normally employed in the routine labora

tory to determine sensitivity, *viz* a 10 per cent blood agar without peptone meat broth (500 g beef heart/1 litre water), glucose 0 3 per cent, NaH_2PO_4 12H O 0 2 per cent, NaCl 0 3 per cent, agar 1 8 per cent, defibrinated horse blood 10 per cent pH adjusted *to 7 3–7 4* *It is* poured into large Petri dishes (14 cm) in a layer of 8 10 mm The plates dry sufficiently during the prediffusion period, so further has been unnecessary

Diffusion Tests

Application to the culture plates of discs medicated with antibiotics A disc prepared *as mentioned* above is placed by means of a sterile tweezer approximately 2 cm from the border of a freshly prepared culture plate (composition see above) and is kept at room temperature (+20° C) for 48 hours at which time an identically prepared disc is placed in a similar manner on the plate, and so forth until 6 discs *are placed on* the same plate allowing diffusion for 96, 48, 24, 8 2 and ½ hours respectively At zero time all the discs are removed Three plates thus prepared are then seeded with 1 1 diluted suspension and 3 with an 1 1000 diluted suspension Readings are performed after incubating at 37° C overnight and the average of 3 *zones is* used for computation of the indices In this way the test is repeated for the antibiotics listed in Table 1

In other hours respectively 2 cm from the bor azole and streptomycin are placed on each of 18 plates and discs of tetracycline chlor amphenicol and erythromycin on another 18 plates All plates are kept at room temperature (+20° C) for 19½ hours at which time similar discs are applied and the diffusion is continued for ½ hour At zero time half the number of the plates are seeded with an 1 1 diluted suspension of bacteria and the other half with an 1 1000 diluted suspension The zone diameters are thus determined three times on different culture plates and the zones with long and short time of prediffusion are determined in the same plate in order to ensure highly uniform experimental environments The average of the three determinations is used in the tables

RESULTS

In the first experiments the inoculum index has been determined, applying six antibiotics in common use to Staphylococcus aureus 209 P (FDA) and varying the time of prediffusion from 30 minutes to 96 hours Table 2 shows the results of these experiments The relationship between inoculum index and predifussion time is found to be dependent to a certain extent of the antibiotics in question A relationship can be demonstrated with penicillin, streptomycin, tetracyclin and erythromycin, since a prolongation of the time of prediffusion decreases the inoculum index

TABLE 2

Relation between Inoculum Index and Prediffusion Time for Staphylococcus aureus *209 P*

Prediffusion time in hrs	Inoculum index in per cent					
	Penicillin 4 I	Sulfathiazole 200 μg	Streptomycin 50 μg	Tetracycline 100 μg	Chloramphenicol 50 μg	Erythromycin 10 μg
96	3 9	16 1	4 1	5 1	25 0	7 1
48	1 3	18 5	4 6	5 7	30 8	7 9
24	5 1	19 8	8 8	7 5	38 3	5 5
8	3 8	27 1	12 2	12 2	29 0	13 7
2	9 8	18 6	17 0	11 8	23 7	20 0
0 5	14 3	31 3	24 4	19 4	23 6	19 9

With sulfathiazole, a decreasing inoculum index is also found with longer periods of prediffusion, but the index is still high, even after prediffusion for 96 hours This is even more *pronounced with chloramphenicol*, where no relationship can be demonstrated between prediffusion time and inoculum index, which is much greater than with the other antibiotics

In order to elucidate the inoculum effect further, it has been decided to determine the inoculum index for a series of bacterial strains with the above mentioned antibiotics, after a short and long prediffusion period respectively The short prediffusion period has been set at 30 minutes, the long period at 20 hours, according to the normal routine in this laboratory Tests have been performed on a total of 47 different strains belonging to 14 genera The tables include those strains for which indices could be calculated, which explains the difference in the number of strains

Tables 3 7 show the results of the tests The mean values are given for the inoculum indices for each genus, together with the highest and lowest values *The penicillin indices are all smaller for all genera after* 20 hours prediffusion (Table 3) The index of the sensitive staphylococci decreases from 18 9 per cent to 7 9 per cent and similar decreases are observed for the other groups of organisms except the penicillin-resistant staphylococci where no change is observed *B coli*, *Salmonella* and *B anitratum* give relatively high indices, possibly because of a weak penicillinase formation in these groups

Tests with sulfathiazole will be described without tables, as numerical values for the indices cannot be given In almost all cases undiluted culture gives growth over the whole prediffused area either there is no inhibition zone at all or the limits of the zone are so irregular and indefinite that measurement is impossible With diluted cultures, very well defined and clear inhibition zones are obtained The relation between inoculum and zone of inhibition is thus very pronounced, and equally so with both short and long periods of prediffusion

In the case of streptomycin, a definite reduction of the inoculum index is found with all groups of organisms examined, with the exception of *B anitratum* (1 strain) (Table 4) With *Staphylococcus aureus* the index decreases from 24 4 per cent to 10 4 per cent, with *B coli* it decreases from 18 2 per cent to 7 5 per cent, and similar reductions are found with the other groups

Similar results are obtained with tetracycline, where *B anitratum* again forms and exception With *Staphylococcus aureus* the index is reduced from 1[illegible] 8 per cent to 4 1 per cent and with *B coli* it is reduced from 14 2 per cent to 4 5 per cent (Table 5)

In the case of chloramphenicol the results are not so distinct (Table 6) With *Staphylococcus aureus* the index increases from 22 3 per cent to 39 8 per cent with a very great variation among the strains If the results with all the organisms are considered as a whole there seems

TABLE 3

Relation between Inoculum Index and Prediffusion Time for Penicillin

Group	No of strains examined	Inoculum bact ml (x 10^8)	Inoculum index in per cent			
			Prediffusion 30 mins		Prediffusion 20 hrs	
			Average	Extremes	Average	Extremes
Staphylococcus aureus (penicillin sensitive)	8	1 7 1 2–2 6	18 9	7 3 – 31 2	7 9	1 4–16 5
Staphylococcus aureus (penicillin resistant)	5	1 1 0 3 1 9	∞		∞	
Sarcina lutea	1	–	18 7		0	
Streptococcus faecalis	2	>10 0	34 2	21 9 – 46 4	7 1	0 –14 1
Listeria monocytogenes	3	1 9	24 9	18 3 – 35 7	11 1	9 7–13 8
Bacillus subtilis	1	0 1	∞		∞	
Bacillus pumilus	1	0 4			9 5	
Corynebacterium diphteriae	4	0 6	∞		∞	
Bacterium coli	5	0 8	27 4	3 6 – 39 9	15 2	10 8–20 7
Salmonella	3	0 9	13 8	0 – 41 4	13 3	6 3–23 8
Pasteurella pseudotuberculosis	3	0 7	20 9	16 5 – 28 3	4 7	1 8– 8 9
Bacterium anitratum	2	–	(120 5)	(36 6)–206 5	53 7	49 0–58 3
Proteus mirabilis	3		20 2	13 2 – 24 3	7 2	2 8–14 6
Proteus morganii	1	5 0	∞		∞	

Determination not performed
∞ Inhibition zone with diluted inoculum and no zone with heavy inoculum
() Zone boundaries blurred difficult to measure

TABLE 4

Relation between Inoculum Index and Prediffusion Time for Streptomycin

Group	No of strains examined	Inoculum bact ml (x 10^9)	Inoculum Index in per cent			
			Prediffusion 30 mins		Prediffusion 20 hrs	
			Average	Extremes	Average	Extremes
Staphylococcus aureus	11	1 6	24 4	8 5 44 5	10 4	1 7-20 0
Sarcina lutea	1	–	3 5		3 3	
Streptococcus faecalis	1	>10 0	3 0		0	
Listeria monocytogenes	3	1 9	23 2	15 6-34 7	7 6	0 –21 2
Bacillus cereus	1	0 2	2 9		2 7	
Bacillus subtilis	1	0 1	26 9		7 8	
Bacillus pumilus	1	0 4	47 4		13 2	
Corynebacterium diphteriae	3	0 6	12 9	11 4-14 3	8 9	5 1-14 9
Bacterium coli	5	0 8	18 2	12 1-24 6	7 5	2 8-10 5
Salmonella	3	0 9	19 6	15 5 22 8	9 6	3 1-14 1
Pasteurella pseudotuberculosis	3	0 7	31 3	15 4 47 8	6 1	0 9-11 7
Bacterium anitratum	1	2 3	16 1		25 6	
Proteus mirabilis	3	–	12 1	1 9-18 2	2 0	0 – 6 1
Proteus morganii	1	5 0	13 5		5 8	

– Determination not performed

TABLE 5

Relation between Inoculum Index and Prediffusion Time for Tetracycline

Group	No. of Strains examined	Inoculum bact./ml (× 10⁹)	Inoculum index in per cent			
			Prediffusion 30 mins.		Prediffusion 20 hrs.	
			Average	Extremes	Average	Extremes
Staphylococcus aureus	10	1.6	15.8	10.9–22.2	4.1	1.4–12.1
Sarcina lutea	1	–	16.3		9.3	
Streptococcus faecalis	1	>10.0	22.0		8.1	
Listeria monocytogenes	3	1.9	16.7	8.6–24.4	8.8	5.9–10.5
Bacillus cereus	1	0.2	7.4		3.2	
Bacillus subtilis	1	0.1	24.2		12.6	
Bacillus pumilus	1	0.4	20.2		8.9	
Corynebacterium diphteriae	3	0.6	18.6	6.2–25.2	7.2	4.0– 9.8
Bacterium coli	5	0.8	14.2	10.3–20.3	4.5	1.6– 6.5
Salmonella	3	0.9	15.7	5.3–22.1	3.4	1.0– 5.6
Pasteurella pseudotuberculosis	3	0.7	19.9	13.5–26.3	7.3	3.1–10.2
Bacterium anitratum	3	1.6	6.9	1.0–13.4	8.4	3.5–13.4
Proteus mirabilis	2	–	8.6	6.9–18.8	3.6	2.4– 8.3
Proteus morganii	1	5.0	20.2		8.8	

– Determination not performed

TABLE 6

Relation between Inoculum Index and Prediffusion Time for Chloramphenicol

Group	No of strains examined	Inoculum bact ml (x 10⁶)	Inoculum Index in per cent			
			Prediffusion 30 mins		Prediffusion 20 hrs	
			Average	Extremes	Average	Extremes
Staphylococcus aureus	13	1 5	22 3	4 2 34 5	33 8	1 5 81 5
Sarcina lutea	1		8 8		8 6	
Streptococcus faecalis	2	>10 0	33 8	17 5 50 0	38 3	31 4 45 2
Streptococcus haemolyticus	1		21 3		13 1	
Listeria monocytogenes	3	1 9	13 7	7 1 21 3	14 3	7 1 22 7
Bacillus cereus	1	0 2	24 2		11 2	
Bacillus subtilis	1	0 1	19 4		5 0	
Bacillus pumilus	1	0 4	16 7		10 8	
Corynebacterium diphtheriae	3	0 6	21 1	13 2 32 6	12 7	12 0 13 2
Bacterium coli	5	0 8	34 4	12 1 85 8	23 9	6 3–64 8
Salmonella	3	0 9	25 0	21 7–31 2	9 8	3 8–14 9
Pasteurella pseudotuberculosis	3	0 7	35 9	23 9 42 9	24 3	8 7–33 0
Proteus mirabilis	2		17 2	15 7 18 6	7 3	5 8–8 8
Proteus morganii	1	5 0	23 2		4 4	

Determination not performed

TABLE 5

Relation between Inoculum Index and Prediffusion Time for Tetracycline

Group	No of Strains examined	Inoculum Bact ml (x 10)	Inoculum index in per cent			
			Prediffusion 30 mins		Prediffusion 20 hrs	
			Average	Extremes	Average	Extremes
Staphylococcus aureus	10	1.6	15.8	10.9 22.2	4.1	1.4–12.1
Sarcina lutea	1	–	16.3		9.3	
Streptococcus faecalis	1	>10.0	22.0		8.1	
Listeria monocytogenes	3	1.9	16.7	8.6 24.4	8.8	5.9 10.5
Bacillus cereus	1	0.2	7.4		3.2	
Bacillus subtilis	1	0.1	24.2		12.6	
Bacillus pumilus	1	0.4	20.2		8.9	
Corynebacterium diphteriae	3	0.6	18.6	6.2 25.2	7.2	4.0– 9.8
Bacterium coli	5	0.8	14.2	10.3 20.3	4.5	1.6– 6.5
Salmonella	3	0.9	15.7	5.3 22.1	3.4	1.0– 5.6
Pasteurella pseudotuberculosis	3	0.7	19.9	13.5 26.3	7.3	3.1–10.2
Bacterium anitratum	3	1.6	6.9	1.0 13.4	8.4	3.5–13.4
Proteus mirabilis	2		8.6	6.9 18.8	3.6	2.4– 8.3
Proteus morganii	1	5.0	20.2		8.8	

– Determination not performed

to be a slight tendency to smaller indices after 20 hours than after 30 minutes of prediffusion

With erythromycin a somewhat smaller number of groups are involved as the Gram negative rods are resistant In the groups examined, there is a uniform decrease in the inoculum index (Table 7) This decrease seems to be more moderate than that found with streptomycin and tetracycline

DISCUSSION

The results give a somewhat heterogeneous picture of the significance of the inoculum, and an unambiguous conclusion is not possible As already suggested a multitude of factors may be responsible for the inoculum effect, for example enzyme formation, the formation of protective substances, substrate competition and diffusion factors A greater or smaller number of these factors will be effective in the individual cases

Prediffusion cannot be expected to influence other factors than those determined by the process of diffusion, so that the penicillinase effect and the p aminobenzoic acid effect must be expected to remain uninfluenced by the diffusion time In accordance with this, prediffusion is found to have no influence on the inoculum effect in these cases With non penicillinase producing strains and penicillin, with streptomycin tetracycline and erythromycin, a uniform reduction in the inoculum effect is found, corresponding to the prolongation of the prediffusion time Prediffusion does not, however, influence the inoculum effect with chloramphenicol

According to Cooper et al (4) and Linton (18), the relationship between inhibition zone and inoculum is given by

$$T = L + G \times \log_2 \frac{N^1}{N_0} \quad (1)$$

where L = lag period G = generation time, N^1 = the critical number, i e bacterial number in a minute area when the zone edge is formed, N_0 = inoculum, i e bacterial number in the same area after seeding and T = critical time The critical time (T) enters in *Cooper & Woodman*'s (5) approximate formula for the standard curve

$$R^2 = 9\,2\ D\,T\ (\log C_0 \quad \log C_n\ ) \quad (2)$$

where R = radius of inhibition zone in mm, D = diffusion constant in mm hr C_0 = antibiotic concentration at the centre at time zero, and C_n = inhibitory concentration in the same units T is thus seen to determine the slope of the standard curve, and N_0 (= inoculum) exerts its effect by altering the value of T and therefore the slope of the standard curve By using the prediffusion technique, formula (1) can be written

TABLE 7

Relation between Inoculum Index and Prediffusion Time for Erythromycin

Group	No of strains examined	Inoculum bact ml (x 10[illegible])	Inoculum index in per cent			
			Prediffusion 30 mins		Prediffusion 20 hrs	
			Average	Extremes	Average	Extremes
Staphylococcus aureus	12	1 5	15 6	4 2 36 0	12 6	0 27 2
Sarcina lutea	1		7 4		4 4	
Streptococcus faecalis	2	>10 0	16 6		14 4	
Streptococcus haemolyticus	1	–	12 6		12 2	
Listeria monocytogenes	3	1 9	24 6	23 2–26 8	9 3	6 1–13 0
Bacillus cereus	1	0 2	16 5		7 4	
Bacillus subtilis	1	0 1	38 2		14 8	
Bacillus pumilus	1	0 4	17 9		18 9	
Corynebacterium diphteriae	3	0 6	20 3	18 5–22 3	8 5	7 4– 9 5

– Determination not performed

inoculum to 1 1000 as a measure of the inoculum effect The results show that prediffusion reduces the inoculum effect, *but with significant exceptions* Thus, in sensitivity tests with penicillin and penicillinase producing organisms, the amount of the inoculum is *decisive* for the size of the inhibition zones formed as in all cases of sensitivity tests using sulfathiazole and chloramphenicol The reasons for these exceptions are discussed

It is concluded that for penicillin, streptomycin, tetracycline and erythromycin the prediffusion method reduces the inoculum effect on the tests and that this fact, in connection with the influence of prediffusion on the correlation between the dilution method and the diffusion method is a further argument for using long prediffusion time in sensitivity tests by the agar diffusion method

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$$T = P + L + G \times \log_2 \frac{N^1}{N_0} \quad (3)$$

where P = time of prediffusion T is thus seen to be increased, so that the slope of the standard curve is also increased, and since P is very great compared with the other terms, the practical significance of the variations in these terms is reduced

In the case of streptomycin, tetracycline, erythromycin and to some extent penicillin, there is good agreement between the anticipated results and those found With sulfathiazole and in other cases with penicillin, the formulae given above are irrelevant Neither of the formulae can be applied in the case of chloramphenicol, no obvious explanation can be found for this It may be that the considerable inoculum effect indicated in the chloramphenicol tests is due to the formation of the enzyme "chloramphenicol reductase" by the bacteria 22) This enzyme has been demonstrated in a number of resistant bacteria, and reductase activity has been suggested as the cause of the chloramphenicol resistance (18) As the inoculum effect, however, *seems to be independent of the sensitivity, this explanation is less* probable It is also conceivable that the chloramphenicol effect is due to inhibition of transport of certain amino acids, so that the effect of the chloramphenicol does not appear until the preformed amount of amino acid is used up (20) While the diffusion-dependent inoculum effect, which is the dominant form with streptomycin, tetracycline and erythromycin, can be reduced by the use of prediffusion, the culture density remains decisive in the case of penicillin (the penicillinase producing strains), sulfonamide and chloramphenicol

The optimal inoculum density remains a matter of discussion In the case of sulfonamides, a too large inoculum will result in a number of sensitive strains being recorded as resistant, and with penicillin a too small inoculum will result in a number of penicillin-resistant strains of staphylococci being reported as wholly or partly sensitive Whether the one mechanisms or the other is operating in the case of chloramphenicol is uncertain and requires investigation

In testing the sensitivity to sulfonamides, it can be recommended that an inoculum should be used which is as dilute as the possibilities for reading allow, then the character of the zone boundaries can be used as a criterion for penicillinase formation, as the penicillinase-producing strains produce irregular boundaries with large marginal colonies, in contrast to the non-penicillinase-producing strains where the colony size decreases gradually (25)

SUMMARY AND CONCLUSIONS

The inoculum effect in bacterial sensitivity tests by the diffusion method is assessed by varying the inoculum at different length of the prediffusion, using the zone increment resulting from a dilution of the

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BRIEF REPORTS

"SANDWICH" TECHNIQUE FOR THE ESTABLISHMENT OF CULTURES OF HUMAN SKIN FOR CHROMOSOME INVESTIGATION

By A J Therkelsen

C for 3-4 days,

is changed and the
concentration of
performed by mea
minutes 5 ml E S
in 0.3 per cent sodium chloride solution incubated at 37° C for 15 minutes and finally fixed in glacial acetic acid methanol exactly as described by Moorhead et al (4)

So far cultures from 67 biopsy specimens have been established by this method without any failures as as least two out of four cultures were always successful

Growth is rapid as the generation time is 16 to 24 hours in the logarithmic growth phase (5) The technique would be ideal as a standard method as culture conditions might be made identical in all laboratories especially if the serum used is taken from the same person as the biopsy specimen

All equipment is comercially available and the cultures can be made by personnel inexperienced in tissue-culture technique

The method has one disadvantage It is impossible to keep the cells growing for more than three months However this is of no great importance as far as most cytogenetical investigations are concerned

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granules

specific antigens Further investigations ...
be adapted for the investigation of Rous virus induced sarcoma in other mammals

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POSSIBLE SURVIVAL FOR YEARS OF LYMPHOCYTES *IN VIVO* WITHOUT MITOTIC DIVISION

By *Jakob Visfeldt*

Within recent years several chromosome workers have demonstrated the presence of various chromosome aberrations, including dicentrics tricentrics and rings in short term cultures of peripheral ... who years ago had been exposed to heavy radiation doses ... vided or they were seen in the fi...
In our chromosome studies on ... treated patients certain features ... f chromosomes are seen actually ... that they can survive for some years in the ... mitotic activity

registration has been made of all of the observed abnormalities

Results In all blood samples from the exposed patients some mitoses were seen with dicentric chromosomes the occurrence declining with time after exposure
A quantitative study showed that *the great majority of cells containing dicentric chromosomes contained also an acentric fragment* (twin fragment double fragment) In cells containing more than one dicentric chromosome there would be one acentric fragment to each dicentric chromosome

Discussion The formation of a dicentric chromosome by fusion of two chromosomes requires that the chromosomes involved are damaged by a break at some site A detached fragment is formed concurrently by the two peripheral chromatin pieces If the damage has occurred prior to the effective duplication of the chromosome an acentric fragment is consequently to be seen, in excess of the dicentric chromosome since also the fragment has divided Recognition of the chromosomes constituting the dicentrics serves for the determination, with some accuracy of

Received 16 iv 64 *from The University Institute for Human Genetics Department of Experimental Genetics and Cytology (J Schult- Larsen M.D) and from The Radium Centre Copenhagen (Professor Jens Nielsen M D)*
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STUDIES IN ROUS RAT SARCOMA WITH FLUORESCENT ANTIBODIES

By *Lars Göran Lindberg*

In recent years fluorescent antibodies against Rous virus have been used in investigations of Rous sarcoma from chickens and of chick fibroblasts infected with Rous virus *in vitro* (*Malmgren et al.* 1960, *Mellors et al.* 1960, *Vogt et al.* 1961). These studies have yielded valuable information on the occurrence and site of the virus. Rous virus induced sarcomas in mammals have been studied for some years at our laboratory. In experiments with fluorescein labelled antibodies against Rous virus attempts were made to demonstrate the virus on frozen sections from Rous virus induced rat sarcoma (RR sarcoma). However, owing to technical difficulties the findings could not easily be evaluated. In the present investigation living suspended sarcoma cells were analysed using the method of *Möller* (1961).

Specific immune serum against Rous virus (Schmidt-Ruppin strain) was prepared by immunizing adult chickens with a partly purified virus fraction either attenuated according to *Mellors et al.* or inactivated with formalin or heat (*Fink et al.* 1961). The starting material consisted of homogenized chicken sarcoma tissue fractioned by differentiated centrifugation according to *Moloney* (1960).

The RR sarcoma was induced about 3 years ago at this laboratory and has since been serially transplanted in rats (96 passages). Finely minced RR sarcoma was injected intraperitoneally into a series of young rats. Ascitic fluid with suspended sarcoma cells was obtained 7–9 days later. The cells were incubated with the immune serum for 20 minutes, washed and then re-incubated with fluorescein labelled rabbit antibodies against chicken gammaglobulin. The cell suspensions were washed again and then analysed and were compared with control suspensions that had been prepared in the same way but incubated with either normal chicken serum or non-specific immune serum from chickens or specific immune serum absorbed with acetone lyophilised chicken sarcoma. The cell suspensions were evaluated in accordance with the criteria given by *Möller* (1961).

TABLE 1

Occurrence of Fluorescent Cells in Rous Rat Sarcoma

Days after intra-peritoneal injection of RR sarcoma	Per cent fluorescent cells in the ascitic fluid after treatment with						
	immune serum		control serum	absorbed serum		anti rabbit albumin serum	
	1:3	1:6		1:3	1:6	1:3	1:6
9	21.1	15.2	1.7				
7	27.0	15.0	2.5			1.8	4.2
9			1.8	1.2	1.7		

Results. Table 1 exemplifies and summarizes some of the experiments. It was found that in about 20 per cent of the RR sarcoma cells the cell membranes contain some substance that reacts specifically to antibodies from the immunized chickens. The partly purified fraction used for immunization contained active virus, for slowly growing tumours developed in some of the immunized chickens. As living cells were used the investigation could only demonstrate specific antigens localized on the surfaces of the cells, since antibodies are considered incapable of penetrating the membranes of living cells. In the earlier investigations of *Mellors et al.* 1960, *Vogt et al.* 1961 on chicken sarcoma and on Rous virus infected fibroblasts large fluorescent aggregates were seen in the cytoplasm near the surfaces of the cells. In the present experiments a homogenous brim of fluorescent material was seen on

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PROTECTION OF C3H MICE AGAINST TOXIC EFFECTS OF ENDOXAN BY MEANS OF CYSTEINE HYDROCHLORIDE

By

HANS BRINCKER

Received 22 xi 63

The purpose of the present experiments has been to investigate the possibility of protecting C3H mice against single toxic doses of endoxan by means of single doses of cysteine hydrochloride Furthermore the connection between the most favourable moment of administering cysteine hydrochloride and the dependence of this moment on some of the pharmacodynamical factors affecting the fate of the two substances investigated in the animal organism

MATERIALS AND METHODS

d into experimental groups each s and females The average weight roups were used in the form of neutral ent The solutions were prepared

njections either subcutaneously in n axillae or intraperiton ally All the an mals used were weighed immediately fore the injections and the d ses then calculated for each animal in proportion t its we ght

TOXIC EFFECTS OF ENDOXAN

Preliminary toxicity experiments with endoxan revealed that the minimal LD_{100} w is 750 mg per kg All the 40 control animals which were treated with this dose died within 6 days The mortality curves were very uniform both for control animals and for other groups of animals identically treated (Fig 1)

It appeared from all the experiments that the tolerance of the females to the toxic effects of endox in was distinctly better than that of the

fr anish C v l Defence Board and an tion and fr m Carl Schepler

w I thank Pr fessor J B chel and D ct rs K Olsen and K B Jensen for their kin l interest help and critici m during the executi n of the present w rk

the size of the acentric fragment Consequently, if radiation damage has affected peripherally both of the chromosomes the size of the formed acentric fragment may be too small for visualization

May a cell which contains a dicentric chromosome plus an acentric fragment have accomplished a series of divisions? The accomplishment of division may be rather complicated for cells with dicentric chromosomes especially the uncoiling phase may be difficult, and bridge formation may occur during anaphase, both of which features are prone to inhibit division For this reason it is hardly possible for cells with dicentric chromosomes to accomplish more than a few divisions An acentric fragment will not become attached to the mitotic spindle It is unlikely that the two halves of the fragment are to be incorporated in individual daughter cells There is reason to believe that acentric fragments are either lost or they are fully incorporated The latter being the case the subsequent division will bring about two morphologically identical acentric fragments To sum up it is a complicated process for a cell with a dicentric chromosome and an acentric fragment to accomplish mitosis, if accomplished the subsequent generation will show generally that the acentric fragment either is lost or it is present in duplo

A closer examination of our material disclosed as mentioned a presence of di*centric chromosomes in cultures from all of the exposed patients The great* majority of cells with dicentric chromosomes was found to contain also an acentric fragment In few cells only such acentric fragment was absent and cells were not encountered in which concomitant acentric fragments were morphologically identical

Conclusion Although a certain reservation is to be taken our knowledge of the mitosis being insufficient it seems to be reasonable to conclude on the basis of the aforementioned considerations that the majority of the cells in the present material containing dicentric chromosomes as well as acentric fragments are seen in the first mitosis following radiation and hence that radiation damaged lymphocytes can persist in the organism for several years without the accomplishment of mitotic division This being the case of lymphocytes we are allowed to suppose that also tumour cells may remain at rest in the body for years without division This may provide an explanation to the fact that metastases can occur several years after a presumed cure

Summary In the course of chromosome studies on peripheral blood from patients exposed to radiation up to six years ago almost all of the mitoses with dicentric chromosomes were found to present also acentric fragments Theoretical deliberations imply that these cells may have persisted since the time of exposure without mitotic division

P S Upon completion of the present report I received a preprint from Edinburgh of a paper by *Buckton* and *Pike* (in press to be published in Cytogenetics) in which similar observations are considered

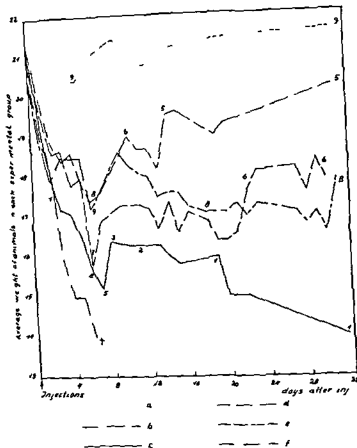


Fig 3

a
d
b
c Cysteine hydrochloride subcut immediately before endoxan subcut
f 1 ml physiological saline subcut immediately before endoxan subcut
e Cysteine hydrochloride subcut 30 minutes before endoxan subcut

males, the females outliving the males both with and without treatment with cysteine hydrochloride Fig 2, showing mortality curves for similarly treated male and female mice, indicates that by means of the mortality curves, the two sexes can be distinguished from each other They were treated at different times with 750 mg endoxan per kg and 720 mg cysteine hydrochloride per kg in various combinations

Autopsies were performed on all dead animals to exclude deaths caused by erroneous injection procedures Such injections might result in subcutaneous haematomas or perforations of internal organs, fol-

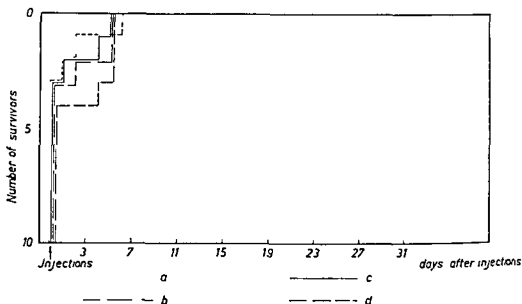


Fig 1

Control curves from 4 different experiments a b, c and d 1 ml physiological saline before 750 mg endoxan per kg

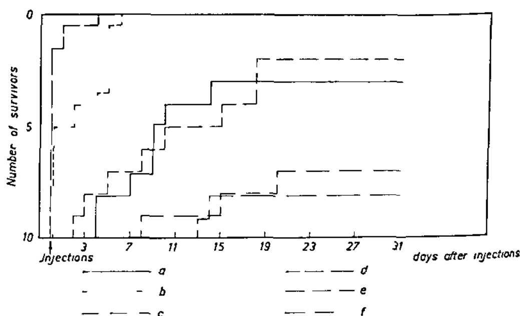


Fig 2

Mortality curves for similarly treated male and female mice

b 20 females, 1 ml physiological saline before 750 mg endoxan per kg (Controls)
d 20 males, 1 ml physiological saline before 750 mg endoxan per kg (Controls)
c 10 females, 720 mg cysteine hydrochloride per kg i p immediately before 750 mg endoxan per kg i p
e 10 males 720 mg cysteine hydrochloride per kg i p immediately before 750 mg endoxan per kg i p
f 10 females, 720 mg cysteine hydrochloride per kg i p immediately before 750 mg endoxan per kg subcut
a 10 males, 720 mg cysteine hydrochloride per kg i p immediately before 750 mg endoxan per kg subcut

lowed by peritonitis *A few animals with these types of findings were* rejected from the experimental material

Protected as well as unprotected animals showed parallel losses of weight after the injections (Fig 3) However, the losses of weight of the best protected animals were less abrupt, and their ensuing increases in weight were more rapid than those of the poorest protected or unprotected animals The maximum loss of weight was reached on the sixth or seventh day after the injections, and animals hereafter not putting on weight soon died

PROTECTIVE EFFECTS OF CYSTEINE HYDROCHLORIDE

Fig 4 shows the results of 2 experiments in which different doses of cysteine were injected, either 20 minutes before or immediately before (*i e* few seconds) 750 mg endoxan per kg In the experiment in which cysteine was injected 20 minutes before endoxan, doses of cysteine hydrochloride of 180 360 and 720 mg per kg respectively were used, and all these injections were given intraperitoneally In the experiment in which cysteine was injected just before endoxan, doses of cysteine hydrochloride of 90, 180 360 and 720 mg per kg, respectively were used, and all the injections in question were given subcutaneously

The control curves of the two experiments are almost identical, and the curves c and h shown in Fig 5 (endoxan and cysteine were injected both subcutaneously and intraperitoneally) are also almost identical Therefore, the results of the two experiments shown in Fig 4 are assumed to be immediately comparable

It appears from the curves that to a certain degree cysteine seems to yield protection against the toxic effects of endoxan, and that this protection is more pronounced when cysteine is injected immediately before endoxan than when it is injected 20 minutes before Furthermore, *it appears from the curves that the protective effect is increased with* increasing doses of cysteine

It is seen that the period of survival of animals receiving 720 mg cysteine hydrochloride per kg 20 minutes before endoxan is shorter than that of animals receiving 180 or 360 mg cysteine hydrochloride per kg immediately *before endoxan This indicates that considerable* quantities of the cysteine—possibly half of it—are eliminated in the course of 20 minutes at any rate as regards its protective effect

In all experiments performed, the doses of cysteine hydrochloride ranged from 90 to 720 mg per kg Therefore it is unknown if it is possible to protect C3H mice completely against the toxic effects of endoxan However in some groups a few mice survived 750 mg endoxan per kg under protection of 720 mg cysteine hydrochloride per kg for more than 4 months Almost all of these animals were females

Fig 5 shows the results of an experiment in which 720 mg cysteine

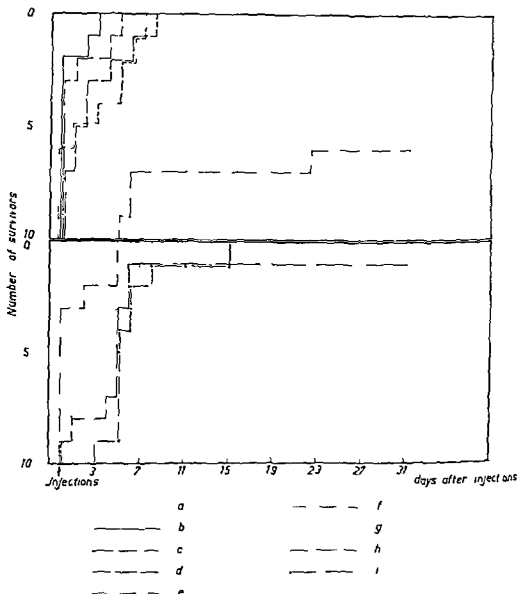


Fig 4

Different doses of cysteine hydrochloride given either 20 minutes before or immediately before the same dose of endoxan

- b 180 mg cysteine hydrochloride per kg i p 20 minutes before 750 mg endoxan per kg i p
- c 1 ml physiological saline subcut immediately before 750 mg endoxan per kg subcut (Controls)
- d 360 mg cysteine hydrochloride per kg i p 20 minutes before 750 mg endoxan per kg i p
- a 90 mg cysteine hydrochloride per kg subcut immediately before 750 mg endoxan per kg subcut
- e 720 mg cysteine hydrochloride per kg subcut immediately before 750 mg endoxan per kg subcut
- f 1 ml physiological saline i p 20 minutes before 750 mg endoxan per kg i p (Controls)
- g 720 mg cysteine hydrochloride per kg i p 20 minutes before 750 mg endoxan per kg i p
- h 180 mg cysteine hydrochloride per kg subcut immediately before 750 mg endoxan per kg subcut
- i 360 mg cysteine hydrochloride per kg subcut immediately before 750 mg endoxan per kg subcut

lowed by peritonitis. A few animals with these types of findings were rejected from the experimental material.

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PROTECTIVE EFFECTS OF CYSTEINE HYDROCHLORIDE

Fig. 4 shows the results of 2 experiments in which different doses of cysteine were injected either 20 minutes before or immediately before (i.e. few seconds) 750 mg endoxan per kg. In the experiment in which cysteine was injected 20 minutes before endoxan, doses of cysteine hydrochloride of 180, 360 and 720 mg per kg respectively were used and all these injections were given intraperitoneally. In the experiment in which cysteine was injected just before endoxan, doses of cysteine hydrochloride of 90, 180, 360 and 720 mg per kg respectively were used and all the injections in question were given subcutaneously.

The control curves of the two experiments are almost identical, and the curves c and h shown in Fig. 4 (endoxan and cysteine were injected both subcutaneously and intraperitoneally) are also almost identical. Therefore the results of the two experiments shown in Fig. 4 are assumed to be immediately comparable.

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It is seen that the period of survival of animals receiving 720 mg cysteine hydrochloride per kg 20 minutes before endoxan is shorter than that of animals receiving 180 or 360 mg cysteine hydrochloride per kg immediately before endoxan. This indicates that considerable quantities of the cysteine—possibly half of it—are eliminated in the course of 20 minutes, at any rate as regards its protective effect.

In all experiments performed the doses of cysteine hydrochloride ranged from 90 to 720 mg per kg. Therefore it is unknown if it is possible to protect C3H mice completely against the toxic effects of endoxan. However, in some groups a few mice survived 750 mg endoxan per kg under protection of 720 mg cysteine hydrochloride per kg for more than 4 months. Almost all of these animals were females.

Fig. 5 shows the results of an experiment in which 720 mg cysteine

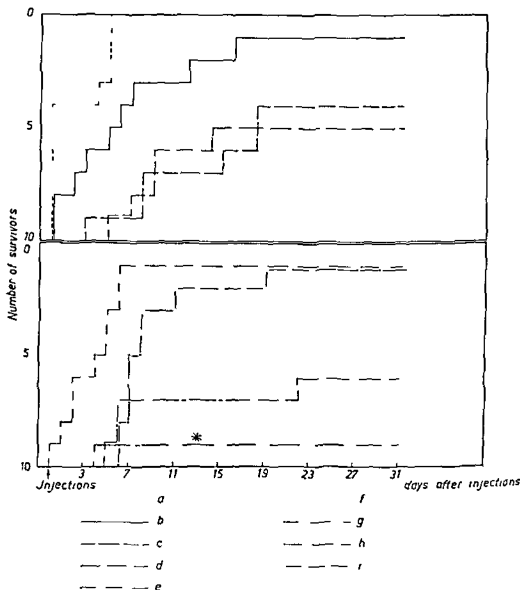


Fig 5

Cysteine hydrochloride injected at different times before endoxan

a 1 ml physiological saline immediately before 750 mg endoxan per kg subcut (Controls)
b 720 mg cysteine hydrochloride per kg i p 1 hour before 750 mg endoxan per kg subcut
c 720 mg cysteine hydrochloride per kg i p immediately before 750 mg endoxan per kg i p
d 720 mg cysteine hydrochloride per kg i p immediately before 750 mg endoxan per kg subcut
e 720 mg cysteine hydrochloride per kg i p 30 minutes before 750 mg endoxan per kg subcut
f 720 mg cysteine hydrochloride per kg subcut 1 hour before 750 mg endoxan per kg subcut
i 720 mg cysteine hydrochloride per kg subcut 30 minutes before 750 mg endoxan per kg subcut
h 720 mg cysteine hydrochloride per kg subcut immediately before 750 mg endoxan per kg subcut
g 720 mg cysteine hydrochloride per kg i p immediately before 1 ml physiological saline subcut (* This animal died of peritonitis)

hydrochloride per kg was injected intraperitoneally or subcutaneously at different times *before* 750 mg endoxan per kg intraperitoneally or subcutaneously

It also appears from these curves that the best protection is obtained by injecting cysteine immediately before endoxan In the groups of animals which had been given injections of cysteine *30 minutes before* endoxan, the specimens which had had cysteine subcutaneously 30 minutes before endoxan subcutaneously seemed to react more favourably than those which had had cysteine intraperitoneally 30 minutes before endoxan subcutaneously

Probably this can be accounted for *by a difference of the speed of* resorption of the cysteine from the peritonaeum and subcutis No clear difference was shown between animals having received cysteine intraperitoneally or subcutaneously 1 hour before endoxan subcutaneously or between animals having received cysteine intraperitoneally or subcutaneously *immediately before endoxan* intraperitoneally or subcutaneously

The figure also shows a group of animals which had only received 720 mg cysteine hydrochloride per kg immediately before physiological saline The only animal in this group, which died after 4 days, died of peritonitis in consequence of an intestinal perforation during the injections None of the other animals, which were observed for more than 4 months, showed any toxic symptoms whatever Frequent weighings *revealed* a constant increase in weight (*See Fig 3*)

Unpublished experiments by *Hastrup* (8), in which mice were subjected to daily intraperitoneal injections of cysteine hydrochloride in doses of 650 mg per kg for more than 3 months, showed that cysteine even in these substantial doses was non toxic Consequently, cysteine must be considered entirely *non-toxic to mice in the doses administered* during the present experiments

Fig 6 shows the results of an experiment in which 720 mg cysteine hydrochloride per kg was injected intraperitoneally or subcutaneously at different times *after* 750 mg endoxan per kg subcutaneously Here too the administration of cysteine seems to be of a distinctly protective effect even as late as 5 hours after the administration of endoxan

A comparison between Figs *5 and 6 shows that cysteine* subcutaneously *30 minutes after* endoxan seems to be as favourable as cysteine immediately before endoxan Cysteine intraperitoneally 30 minutes and 1 *hour after* endoxan is nearly as favourable as cysteine immediately before endoxan Cysteine subcutaneously 1 hour after endoxan and cysteine intraperitoneally 2 hours after endoxan seems to give slightly less favourable but otherwise identical results Cysteine subcutaneously 2, 3 and 4 hours after endoxan seems to give almost identical results

The somewhat different results of *intraperitoneal and subcutaneous* injections of cysteine at the same times after the administration of

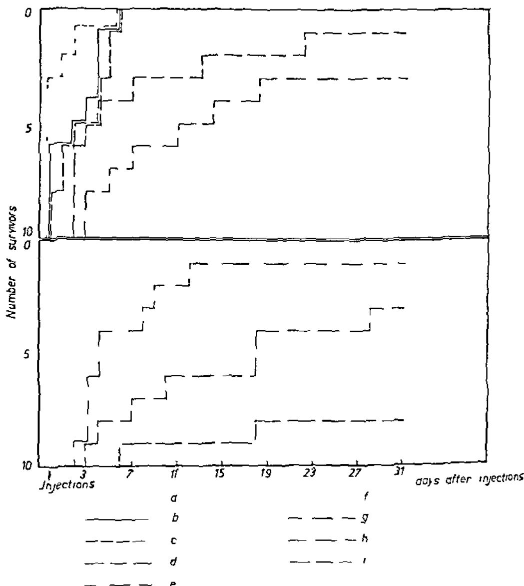


Fig 6

Cysteine hydrochloride injected at different times after endoxan

a 1 ml physiological saline immediately before 750 mg endoxan per kg subcut (*Controls*)
b 720 mg cysteine hydrochloride per kg subcut 3 hours after 750 mg endoxan per kg subcut
c 720 mg cysteine hydrochloride per kg subcut 3 hours after 750 mg endoxan per kg subcut
d 720 mg cysteine hydrochloride per kg i p 2 hours after 750 mg endoxan per kg subcut
e 720 mg cysteine hydrochloride per kg i p 1 hour after 750 mg endoxan per kg subcut
f 720mg cysteine hydrochloride per kg subcut 2 hours after 750 mg endoxan per kg subcut
g 720 mg cysteine hydrochloride per kg subcut 1 hour after 750 mg endoxan per kg subcut
h 720 mg cysteine hydrochloride per kg i p 30 minutes after 750 mg endoxan per kg subcut
i 720 mg cysteine hydrochloride per kg subcut 30 minutes after 750 mg endoxan per kg subcut

endoxan may be ascribed to differences of resorption from the peritonaeum and subcutis, cf Fig 5

Of animals treated with cysteine 5 hours after endoxan, 2 out of 10 died within 5 hours after the injections of endoxan Consequently, it was not tried to administer cysteine later than 5 hours after endoxan The difference between the results of groups of animals being treated with cysteine 2, 3 and 5 hours after endoxan is remarkably slight It is [illegible] that a protection of the animals—however modest—may be [illegible]

of a radioprotective agent

DISCUSSION

Apparently, only few investigations of the effects of combined treatment with endoxan and radioprotective compounds have been performed

Haas (6) investigated whether beta-aminoethylthiouroniumchloride hydrochloride (AET) protected rats against toxic doses of endoxan, but found no protection, though the compound in similar experiments was found to give protection against toxic doses of nitrogen mustard (HN2)

By combined treatment of homologous mouse tumours with endoxan and cysteamine *Hastrup* (7) showed that this combined treatment gave better results (as measured by the period of survival of the animals) than treatment of the same tumours with endoxan only

In experiments in which rats were give injections of 1000 mg cysteine hydrochloride per kg intraperitoneally 30–45 minutes before 250 mg endoxan per kg subcutaneously, *Balla et al* (2) were unable to demonstrate any protective effect of cysteine against the lethal effects of endoxan, in so far as the animals thus treated died as quickly as animals receiving endoxan only in the said dose

Contrary to this, in experiments with AKA mice receiving daily intraperitoneal injections of endoxan in doses of 2, 3 and 4 mg per mouse, *Hastrup* (8) demonstrated an unmistakable prolongation of the period of survival of the animals when at the same time they received daily intraperitoneal injections of cysteine hydrochloride in doses of 2 28 13 68 mg per mouse 15 minutes before the injections of endoxan The protective effect of the cysteine increased with increasing doses of cysteine, and the compound did not give rise to toxic symptoms when given solely intraperitoneally in the said daily doses for 3 months

In all essentials, the present investigations arrive at the same conclusions as *Hastrup* (8) Their most important results are 1) that, altogether, cysteine protects against the toxic effects of endoxan and 2) that this protection can be obtained by the administration of cysteine both before and after the administration of endoxan

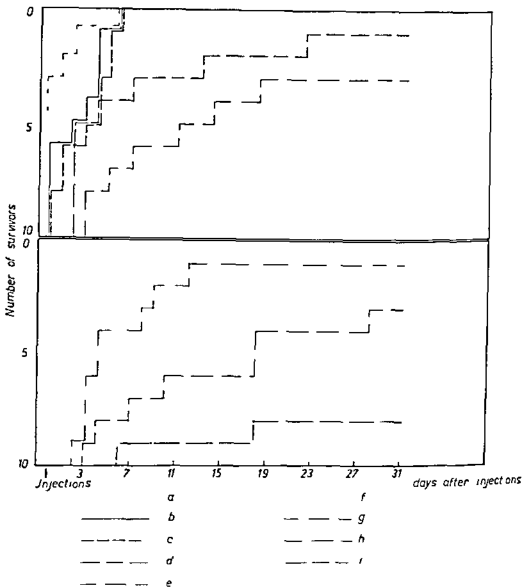


Fig 6

Cysteine hydrochloride injected at different times after endoxan

a 1 ml physiological saline immediately before 750 mg endoxan per kg subcut (Controls)
b 720 mg cysteine hydrochloride per kg subcut 5 hours after 750 mg endoxan per kg subcut
c 720 mg cysteine hydrochloride per kg subcut 3 hours after 750 mg endoxan per kg subcut
d 720 mg cysteine hydrochloride per kg i p 2 hours after 750 mg endoxan per kg subcut
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h 720 mg cysteine hydrochloride per kg i p 30 minutes after 750 mg endoxan per kg subcut
i 720 mg cysteine hydrochloride per kg subcut 30 minutes after 750 mg endoxan per kg subcut

chloride per kg intraperitoneally immediately before 750 mg endoxan per kg intraperitoneally, by which procedure the compounds were mixed in the peritoneal cavity As the molar concentration of cysteine is to that of endoxan as 3 is to 2, and as 6 out of 10 animals from this group died within one month, this at least tells against a direct mole to mole interaction between cysteine and the transport form of endoxan, as in such case one would expect all the animals in the group to survive

On the basis of the experiments performed it is considerably more difficult to decide whether a direct mole to mole interaction takes place between cysteine and the active form of endoxan As appears from Figs 4 and 5 the elimination of cysteine proceeds very rapidly, which means that a large portion of it will be eliminated before all the endoxan has become activated This means that a mole to mole interaction between cysteine and the active form of endoxan, if any, will not be manifested in an increased tendency to survive among the animals treated

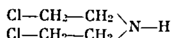
These two factors combined *The slow activation of the endoxan and the rapid elimination of the cysteine* may probably explain the negative result of the experiments of *Balla et al* (2) to protect rats against the effects of lethal doses of endoxan by means of cysteine hydrochloride—especially so if in rats the elimination of cysteine takes place more rapidly and the activation of endoxan more slowly than in mice

It is difficult to explain why in all experiments with mice the males died before the females in spite of the fact that each animal got the doses computed per weight unit of both endoxan and cysteine hydrochloride in exact proportion to its individual weight As the males were a few grams heavier than the females of the same age group, one would think that the difference in death rates might be due to the fact that the males got proportionally too large doses of endoxan on account of their more pronounced fatty tissue This assumption cannot, however, be correct In 19 differently treated experimental groups, each of 10 mice one female, at least, in each group weighed as much or more than the smallest male in the same group Only in 3 of these groups the female died before the male It must therefore be concluded that a real difference of tolerance to endoxan exists between males and females, the females being the more resistent

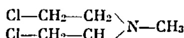
SUMMARY

The present experiments have established that to a certain degree it is possible to protect C3H mice against the toxic effects of endoxan by means of cysteine hydrochloride, even when the latter is injected after endoxan A hypothesis has been advanced to explain this phenomenon Furthermore the experiments have given some basis for an evaluation of the activation time of endoxan *in vivo*, and of the elimination time of cysteine as regards radioprotective properties

As compared with other cytotoxic compounds, endoxan is characterized by being inactive *in vitro* towards cells in tissue cultures In order to activate its toxic properties, it must be introduced into an animal organism It is assumed (3, 5) that endoxan represents a transport form, inactive in itself, from which the active form only develops in the organism, possibly through a disintegration by means of a phosphamidase In hydrolysis experiments *in vitro* with endoxan it has been shown that one of the products of the hydrolysis



being to a certain degree similar to HN2



has cytostatic properties, as has the latter (1)

It has been demonstrated by *Desaive & Varetto-Denoel* (4) and by *Therkelsen* (9) that cysteamine, which acts radioprotectively in a manner similar to cysteine, does not protect experimental animals against the toxic effects of HN2 when administered after the latter, whereas cysteamine in doses of 75–150 mg per kg has an excellent protective effect against the toxic effects of HN2 when administered from 0–30 minutes before HN2

The following hypothesis is advanced to explain author's demonstration of the possibility of protecting mice against the toxic effects of endoxan by administering cysteine *after* the former

It is assumed that the activation of endoxan from transport form to active form takes a certain time so that the full toxic effect is obtained only by degrees Provided that the active form of endoxan is at once linked to its point of attack in the living cell, no protection may be expected against the amount of endoxan which has already been transformed into its active form when the cysteine in injected On the other hand, the conclusion may be drawn that the amount of endoxan activated after the injection of cysteine is somehow prevented from exercising its entire toxic effects by the latter The result is a prolonged period of survival of the animals thus treated

Accordingly the experiments described in Fig 6 probably indicate that the total time of activation of the dose of endoxan used during the experiments must be more than 5 hours, but the main portion of the endoxan is probably activated between 2 and 3 hours after the injection

The experiments performed give no definite information as to the interesting question whether the protective effect of cysteine depends on a simple molecular interreaction between cysteine and endoxan, on a competitive inhibition, or on a more specific form of protection on the cellular level

As shown in Fig 5, a group of mice was given 720 mg cysteine hydro-

chloride per kg intraperitoneally immediately before 750 mg endoxan per kg intraperitoneally by which procedure the compounds were mixed in the peritoneal cavity As the molar concentration of cysteine is to that of endoxan as 3 is to 2 and as 6 out of 10 animals from this group died within one month this at least tells against a direct mole to mole interaction between cysteine and the transport form of endoxan as in such case one would expect all the animals in the group to survive

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These two factors combined *The slow activation of the endoxan and the rapid elimination of the cysteine* may probably explain the negative *result of the experiments of Balla et al (2) to protect rats against the* effects of lethal doses of endoxan by means of cysteine hydrochloride—especially so if in rats the elimination of cysteine takes place more rapidly and the activation of endoxan more slowly than in mice

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SUMMARY

is [illegible] ain degree it [illegible] endoxan by means of cysteine hydrochloride even when the latter is injected after endoxan A hypothesis has been advanced to explain this phenomenon Furthermore the experiments have given some basis for an evaluation of the activation time of endoxan *in vivo* and of the elimination time of cysteine as regards radioprotective properties

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STUDIES ON OXIDATIVE ENZYME SYSTEMS OF THE RAT KIDNEY AFTER HAEMOLYSIS AND *HAEMOGLOBINURIA*

By

GUSTAV DALLNER and JAN L E ERICSSON

Received 4 xii 63

The renal lesion following acute haemolytical conditions in man, such as blackwater fever" and mismatched transfusions, has morphologically been termed "lower nephron nephrosis" or "haemoglobinuric nephrosis", because numerous pigment casts are usually found in the distal parts of the nephron, which in addition show various degenerative changes in the epithelium

Although hypoxaemia and renal tubular anoxia seem to be important factors the pathogenesis of the kidney damage has not been elucidated The question whether the filtered haemoglobin has a damaging effect on the tubular cells of the kidney has been much debated in both clinical and experimental investigations and the results are contradictory (*Yuile* 1942, 1945, *Flink* 1947, *Rather* 1948, *Maluf* 1949, *Hampton & Mayerson* 1950, *Lalich & Schwartz* 1950, *Zingg & Zollinger* 1951, *Block et al* 1952, *Lalich* 1955 a, b, *Finckh* 1957, *Fajers* 1958, 1959 a, b)

The use of the electron microscope and recent developments in histochemical techniques, making it possible to localize many important enzymes on a cytological level, provide new tools for the study of cellular reactions to injury In order to elucidate the pathogenesis of the renal changes during haemoglobinuria and haemolysis, we have performed a series of experiments in which the tubular alterations have been studied with electron microscopic, biochemical and histochemical techniques Some of the preliminary results of these studies have been reported elsewhere (*Dallner & Ericsson* 1962, *Ericsson & Dallner* 1962, *Ericsson* 1962)

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In these experiments the effect of intravenously injected homologous haemoglobin on the kidney was studied The effect of various degrees of chemically induced haemolysis was investigated after subcutaneous injection of glycerine (*Cameron & Finckh* 1956) or intravenous injection of a surface active detergent, sodium deoxycholate (NaDOC) (*Campbell* 1961). With these methods haemolysis and haemoglobinuria, as well as a model for the study of acute renal failure were obtained (*Campbell* 1961)

The present investigation, in which 12 oxidative enzymes were studied histochemically, revealed, that these enzyme systems in the renal proximal tubular cells during the absorption and breakdown of homologous haemoglobin were not affected If, however, the haemoglobinuria was due to haemolysis induced with glycerine or NaDOC, the effect on the tubular enzymes was complex and probably at least partially related directly to the injected substances It appeared that the primary cause of the tubular damage after severe haemolysis induced with a large dose of glycerine was the result of anoxia, since neither haemoglobin nor glycerine were nephrotoxic

MATERIALS AND METHODS

Rats In all experiments the inbred strain R/StDa (strain R subline Stockholm Dallner) was used [1]

A strict, single lined brother sister mating was used to maintain the breeding nucleus The distance among relatives was not more than second cousins

One hundred and twenty young male and female rats 4 to 6 months of age and weighing 250–350 g were used for the experiments The animals received drinking water and the standard diet *ad libitum*

Induction of haemoglobinuria This was performed in three different ways 1) Injection of glycerine solution subcutaneously 2) Injection of NaDOC intravenously 3) Injection of homologous lyophilized haemoglobin solution intravenously

1) A 50 per cent double distilled solution of glycerine in sterile 0 85 per cent NaCl was used It was administered subcutaneously in two different doses a) 0 4 ml/100 g of body weight (b w) = 'small dose of glycerine b) 1 75 ml/100 g of b w = large' dose of glycerine

2) 0 75 ml/100 g of b w of a 0 5 per cent solution of NaDOC in distilled water was given slowly intravenously

3) A 16 per cent solution of lyophilized haemoglobin in double distilled water was given intravenously in a dose of 300 mg haemoglobin/100 g of b w

Control animals in the different experiments were given sterile 0 85 per cent saline instead of the injected substances or did not receive any injection at all

In another type of control experiment designed to clarify the effect of glycerine after different ways of administration the substance was given as a 50 per cent solution in sterile 0 85 per cent saline intraperitoneally subcutaneously or intravenously in a dose of 1 0 ml 100 g of b w

All intravenous injections were made in the left femoral vein When the intravenous and intraperitoneal injections of glycerine were performed care was taken to avoid leakage of the solutions into the subcutaneous tissues All intravenous injections were made under light ether anaesthesia

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1 The breeding pairs were obtained from *Dr O Mühlbock* (Institut Neerlandais Du Cancer Amsterdam Holland) by courtesy of *Dr G Bloom* (Stockholm Sweden) This strain was started and inbred through 28 generations in brother sister matings by Dr Muhlbock

Lyophilization of haemoglobin Blood was withdrawn by heart puncture with a

4° C under sterile conditions Lyophilization was then made in high vacuum at lowered temperature for 24 hours

Biochemical Assay

2 *Respiration and oxidative phosphorylation* The mitochondrial fraction was prepared as was described for measurements of diaphorase activities, and the whole kidney was homogenized

tassium orthophosphate (pH 7 5) 2 μmoles disodium adenosine triphosphate, 4

Histochemical Methods

The experimental animals together with appropriate controls were sacrificed by decapitation 2 or 24 hours after the injection was given In addition, 4 rats were sacrificed 48 and 96 hours after a large dose of glycerine, 2 animals at each time Immediately after death kidney tissue was removed for both conventional light microscopic investigation and cryostat sectioning for histochemistry For the histochemical studies 2 4 mm thick slices of the kidneys were rapidly frozen 4 to 8 μ thick were cut on a System medium contained 0 4 mM n BT 15 mM azide 0 05 M K-phosphate pH 7 5 with d with TPN linked substrates 0 7 mM TPN, and a substrate in a final volume of 1 5 ml The concentration of substrates were DPNH and TPNH 0 6 mM glucose 6-phosphate, 5 mM, 6 phosphogluconate, isocitrate and α glycerophosphate 10 mM, glutamate malate ethanol and lactate, 0 1 M DL β hydroxybutyrate 0 3 M and succinate 0 4 M The incubation times for

malin and mounted in glycerine jelly

RESULTS

A *The Effect of Subcutaneous Injection of Glycerine, and Intravenous Injection of NaDOC and Lyophilized Rat Haemoglobin*

The degree of haemoglobinaemia after intravenous injection of lyophilized haemoglobin or haemolysis induced with glycerine or NaDOC is shown in Fig 10 (page 341) The rate of elimination of the haemoglobin after a large dose of glycerine showed considerable variation among the animals, depending on the degree and time of onset of renal impairment during the initial 24 hour period (*Ericsson* 1964)

1 *Histologic Changes*

These changes will be described elsewhere (*Ericsson* 1964), but in order to provide a basis for the histochemical studies in the present investigation they will be shortly summarized here Thirty minutes after the injection of the large dose of glycerine subcutaneously an accumulation of haemoglobin absorption droplets was observed in the cells of the proximal convoluted tubules After 8 hours the droplets were very numerous, almost completely filling the cytoplasm of these cells, and some droplets were also present in the cells of the straight parts of the proximal tubules Twenty to twenty-four hours after the injection many of the proximal convoluted tubules appeared necrotic The tubules of the distal parts of the nephron were dilated, containing numerous haemoglobin casts, but the cells did not reveal degenerative changes

The intracellular accumulation of haemoglobin absorption droplets in the proximal convoluted tubules following the intravenous injection of homologous haemoglobin was most pronounced at 4 hours, roughly corresponding to the amounts seen 8 hours after the injection of the large dose of glycerine A slight haemoglobin absorption was also noted in the straight parts of the proximal tubules The numbers of droplets then rapidly decreased, and at 8 hours, when the haemoglobinuria had ceased, only occasional droplets were present in the proximal tubules Similar changes were observed after NaDOC or the small dose of glycerine, although smaller amounts of haemoglobin were absorbed in the proximal convoluted tubules and almost none in the straight parts Tubular necrosis was not observed after injection of homologous haemoglobin, NaDOC or the small dose of glycerine

2 *Histochemical Changes*

The special topographical anatomy of the rat kidney permits the localization of different parts of the tubules in a histological section (*Sternberg et al* 1956) Four distinct zones are distinguished (Fig 14) the outer cortex, mainly composed of the proximal convoluted tubules, the inner cortex, in which the straight parts of the proximal tubules predominate, the outer medulla, containing mainly the thick, ascending limbs of Henle's loops, and the inner medulla, in which col-

lecting ducts and the thin limbs of Henle's loops predominate This orderly arrangement facilitates the study of oxidative enzymes, as the activities vary considerably in different parts of the nephron

For the convenience of presentation the findings will be described in two parts In Part I some patterns of enzyme reactions have been selected which serve to illustrate characteristic changes in the different experiments, and form a basis for Part II, in which a summary of the alterations of the 12 oxidative enzymes is given

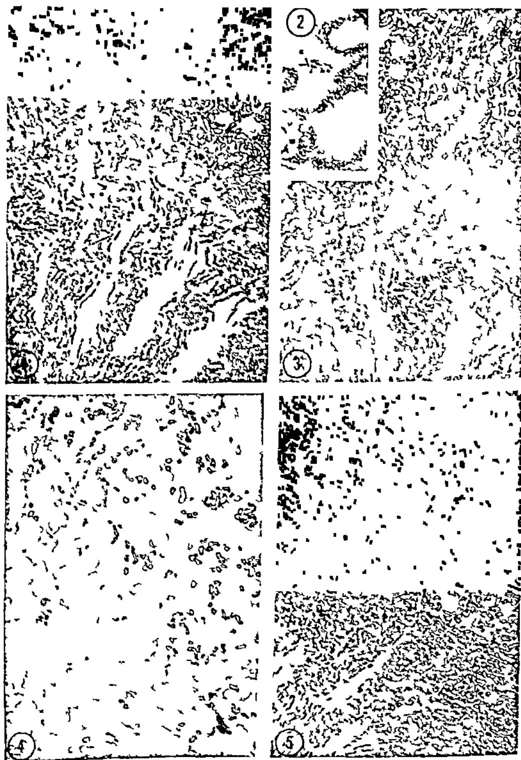
PART I PATTERNS OF REACTION

a *Controls* Fig 1 shows the normal distribution of DPNH diaphorase in the rat kidney The enzyme activity was high in the outer cortex in which the proximal and distal convoluted tubules are located There was also pronounced formazan deposition in the outer medulla, corresponding to the thick, ascending limbs of Henle's loops On the other hand the straight parts of the proximal tubules in the inner cortex had a low enzyme activity The formazan deposit was finely granular (Fig 2)

b *Diminished formazan deposition* Twenty four hours after the subcutaneous injection of a large dose of glycerine there was a pronounced decrease of enzyme activity both in the cortex and the outer medulla as indicated by a diffuse reduction of the granular formazan deposits within the cells (Fig 3) Four days after the injection there was almost no DPNH diaphorase activity in the cortex with the exception of the distal convoluted tubules and the collecting ducts (Fig 4)

c *Increased formazan deposition* The distribution and reaction of DPNH diaphorase was the same as in control kidneys both 2 and 24 hours after the injection of NaDOC with but one exception there was a marked increase in formazan formation in the inner cortex (Fig 5) This kind of change (corresponding to the straight parts of the proximal tubules) after haemoglobinuria caused by NaDOC was even more evident when DL-β hydroxybutyrate dehydrogenase an enzyme involved in fatty acid metabolism was investigated The strongest formazan deposition caused by the activity of this enzyme is normally found in the distal convoluted tubules and the thick, ascending limbs of Henle's loops (Fig 6) Both 2 and 24 hours after the induction of haemolysis and haemoglobinuria with NaDOC there was a striking increase in staining intensity of the inner cortex (Fig 7)

d *Unchanged formazan deposition* Although some of the oxidative enzymes in the kidney showed a very pronounced decrease in formazan formation after injection of the large dose of glycerine, others were not changed α glycerophosphate dehydrogenase an enzyme involved in glycolysis normally shows the strongest reaction in the inner cortex (Fig 8) Twenty four hours after the large dose of glycerine the reaction was about the same as in the controls (Fig 9)


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5

PART II SUMMARY OF THE CHANGES

A summary of the activities of the 12 different oxidative enzymes in the proximal tubules is given in Figs 11 13

The degree of alteration of formazan deposition in the 4 different zones of the kidney was *estimated* in a semiquantitative way by the use of a scale from 0 to 4 The mean value after estimation of the changes in *3 different animals at each time* period was calculated and expressed in per cent of the activity observed in the controls No appreciable difference in the staining reactions was observed among the control animals (males and females) receiving 0 85 per cent NaCl or no injection at all The normal distribution of the enzymes studied was in agreement with what has been described earlier (*Wachstein* 1955, *Farber et al* 19 ... *Sternberg et al* 1956 *Hess et al* 1958, *Pearse* 1960)

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and the straight parts of the tubules The ascending limbs of Henle's loops in general showed less pronounced changes than the proximal tubules In the distal convoluted tubules and collecting ducts there were no appreciable variations between controls and experimental animals The faint enzyme reactions in the thin limbs of Henle's loops both in control and experimental animals made it hazardous to estimate changes in this part of the nephron with certainty As judged by formazan deposition, there was a marked decrease in the activities of the most important energy yielding enzymes, namely respiratory chain enzymes (TPNH and DPNH diaphorase) and three of the histochemically demonstrable catalysts of the citric acid cycle (succinate, malate and isocitrate dehydrogenase) The pentose phosphate pathway, the glycolysis and the glutamate dehydrogenase showed small or no changes

b NaDOC and small dose of glycerine The histochemical activities

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Figs 1 5

Fig 1 DPNH diaphorase activity in normal rat kidney Note the weak activity in the inner cortex which is *in sharp contrast to the reaction in* tubules in the outer cortex and outer medulla × 25

Fig 2 High magnification of some of the thick limbs of Henle's loops Same section as in Fig 1 The *reaction product* is finely granular × 485

Fig 3 DPNH diaphorase activity 24 hours after the large subcutaneous dose of glycerine Strong general decrease in formazan deposition × 25

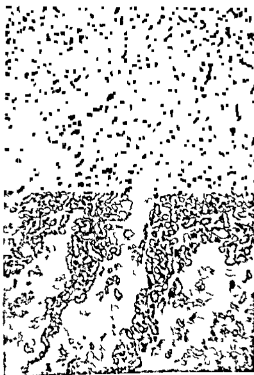
Fig 4 DPNH diaphorase *reaction 4 days after* the large subcutaneous dose of glycerine Formazan formation only in distal tubules and collecting ducts. × 25

Fig 5 DPNH diaphorase reaction 24 hours after intravenous injection of NaDOC Note *the strong increase in formazan* deposition in the straight part of the proximal tubules as compared with Fig 1 × 25

The photomicrographs have all been arranged in such a way that the outer cortex appears in the right upper part of the picture and the outer medulla in the left lower part The inner medulla is not shown


6


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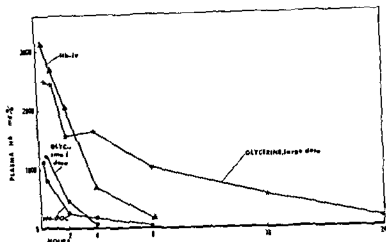


Fig 10

Amount of free haemoglobin in blood plasma after small and large dose of glycerine subcutaneously and intravenous injection of NaDOC and homologous haemoglobin at different time intervals after the injection

of the above mentioned enzymes in the straight parts of the proximal tubules after haemoglobinuria caused by intravenous injection of NaDOC are summarized in Fig 12 The same type of reaction was seen also in the proximal convoluted tubules, although less pronounced The histochemical reactions for practically all of the oxidative enzymes revealed a more or less pronounced increase

The small dose of glycerine, which induced haemolysis of a similar degree as NaDOC, caused slight changes in some of the enzyme activities in the straight parts of the proximal tubules (Fig 13) Contrary to the effect of NaDOC, only some enzymes showed alteration after injection of glycerine Although there was some increase in formazan formation as compared with the controls, it was not at all as great as following NaDOC treatment, and furthermore, the changes appeared later

c *Intravenous injection of lyophilized haemoglobin* The amount of haemoglobin injected (300 mg/100 g of b w) was selected with the pur-

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Figs 6–9

Fig 6 β hydroxybutyrate dehydrogenase in normal kidney Reaction product only in the distal convoluted tubules and the thick limbs of Henle s loops in the outer medulla × 30

Fig 7 β hydroxybutyrate dehydrogenase 24 hours after intravenous injection of NaDOC Extensive formazan deposits in the straight parts of the proximal tubules in the inner cortex × 30

Fig 8 α glycerophosphate dehydrogenase in normal kidney Strongest formazan formation in the straight parts of the proximal tubules × 30

Fig 9 α glycerophosphate dehydrogenase 24 hours after large subcutaneous dose of glycerine No noteworthy changes in comparison with Fig 8 × 30

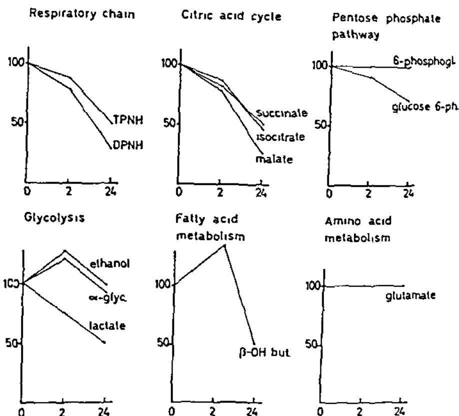


Fig 11

Relative histochemical activity of oxidative enzymes in the proximal tubules (convoluted and straight parts) after large (1 75 ml/100 g of b w) dose of glycerine given subcutaneously The enzyme activity (estimated values) expressed in per cent is given on the ordinate (the activity at the time 0 is 100 per cent and is that observed in the control animals) The time on the abscissa is not drawn in a proportional way The name of appropriate substrates are used as abbreviations for the different dehydrogenases

pose of inducing haemoglobinuria of the same order as after a large dose of glycerine (*Ericsson* 1964) Histochemical study of the activity of oxidative enzymes 2 and 24 hours after the injection did not reveal any changes, *i e* the reactions were of the same intensity in all parts of the nephron as compared to control animals

B *The Effect of Intravenous and Intraperitoneal Injection of Glycerine*

A dose of 1 0 ml of a 50 per cent glycerine solution 100 g of b w was selected for this part of the study, as preliminary experiments had shown that if larger doses were given some of the animals died shortly after the injection The effect of the intravenous and intraperitoneal injection of this dose was compared with that of the subcutaneous injection of the same amount The intravenous injection of glycerine caused a slight haemolysis with plasma haemoglobin ranging from 147 to 486 mg per cent The intraperitoneal injection, on the other hand, induced a much more severe haemolysis with plasma haemoglobin va-

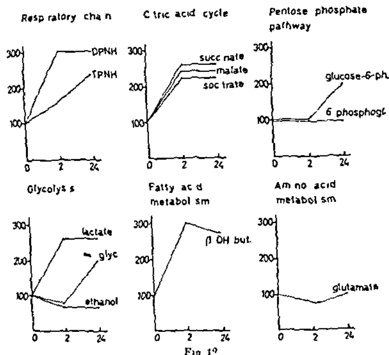


Fig 19

Relati e hi tochemical activity of oxidative enzymes in the straight parts of the prox mal tubules after intravenous injection of NaDOC (0 75 ml/100 g) Otherwise as in Fig 11

lues up to 1800 mg per cent No morphologic lesions or changes in the oxidative enzymes were observed after the intravenous injections of glycerine whereas the same amount given intraperitoneally induced tubular changes similar to those seen after subcutaneous injection The animals given glycerine intraperitoneally showed signs of severe peri tonitis

C *Biochemical Control Studies*

1 *Large Dose of Glycerine*

The diaphorase activities in different cell fractions of the kidney cortex were determined after the large dose of glycerine (Table 1) Both mitochondria and microsomes showed a pronounced decrease of enzyme activities measured with DCPIP These diaphorases showed practically no dicoumarol sensitivity The supernatant fluid contained a relatively high activity of the non specific highly dicoumarol sensitive DT diaphorase isolated by Ernster (*Ernster* 1958 *Ernster et al* 1960 1962) from rat liver This enzyme which does not react with tetra z lium salts did not show diminished activity as opposed to diaphorases in the particulate fractions

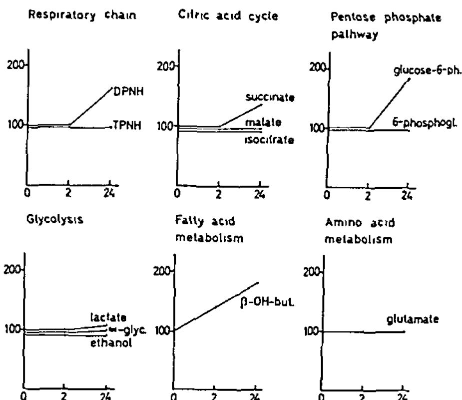


Fig 13

Relative histochemical activity of oxidative enzymes in the straight parts of the proximal tubules after small (0 4 ml/100 g) dose of glycerine given subcutaneously Otherwise as in Fig 11

TABLE 1

Diaphorase Activities in the Kidney Cortex Fractions Measured with DCPIP

	Experiment No	Mitochondria DPNH	Mitochondria TPNH	Microsomes DPNH	Microsomes TPNH	Supernatant DPNH or TPNH
Control	1	1 05	0 17	0 44	0 08	1 32
	2	1 11	0 22	0 57	0 13	1 26
Glycerine 2 hrs	1	0 67	0 15	0 30	0 05	1 22
	2	0 80	0 15	0 35	0 11	1 14
Glycerine 24 hrs	1	0 46	0 08	0 11	0 05	1 55
	2	0 30	0 10	0 19	0 00	1 83

Fractionation procedures and measurements of enzyme activities as described in Materials and Methods Activity in the supernatant which was the same for both the pyridine nucleotides only represents the dicoumarol sensitive part that is not the endogenous activity Values expressed in terms of μmoles DPNH or TPNH oxidized per minute and gram wet weight

2 *NaDOC and Small Dose of Glycerine*

Intravenous injection of NaDOC and subcutaneous injection of the small dose of glycerine resulted in increased formazan deposits in the

proximal tubules This effect was most evident in the case of DL β hydroxybutyrate dehydrogenase, which in the control animals showed a very faint activity in this part of the nephron (Fig 6) and a pronounced increase after NaDOC (Fig 7) Therefore an investigation of the respiration of isolated mitochondria with DL-β hydroxybutyrate as substrate was performed The β hydroxybutyrate oxidase activity was not increased after NaDOC treatment of the rats (Table 2) The oxygen consumption of the mitochondria was the same before and after the injection of NaDOC and the P O ratios were unaltered No respiration occurred in the absence of terminal phosphate acceptor indicating that *there was a full respiratory control*

TABLE 2

Respiration and Oxidative Phosphorylation of Isolated Kidney Mitochondria with DL β Hydroxybutyrate as Substrate

Exp No	Treatment	Respiration μatoms oxygen	Phosphorylation μmoles phosphate	P O
1	none	11 5	20.3	1 8
	none without phosphate acceptor	0 4		
2	none	13 2	21 5	1 6
	none without phosphate acceptor	0		
3	none	9 8	15 1	1 6
	none without phosphate acceptor	0 7		
4	NaDOC	9 6	16 1	1 7
	NaDOC without phosphate acceptor	0 5		
5	NaDOC	11 7	18 1	1 5
	NaDOC, without phosphate acceptor	0 5		
6	NaDOC	10 5	18 6	1 8
	NaDOC, without phosphate acceptor	0 9		

Concerning fractionation procedure measurements and calculations see Materials and Methods

DISCUSSION

Investigations of the reactions of tetrazolium salts with succinate as substrate demonstrated that these electron acceptors do not react with the succinic dehydrogenase but with one or a number of intermediate components in the respiratory chain (*Nachlas et al* 1960 *Wattenberg & Leong* 1960 *Tester & Smith* 1961) If there is also an intermediate between the pyridine nucleotide oxidizing flavoproteins and the tetrazolium salt leakage of this intermediate may cause a diminished histochemical reaction without a decrease of diaphorase activities Recently

salt The present investigation on kidneys from rats given the large

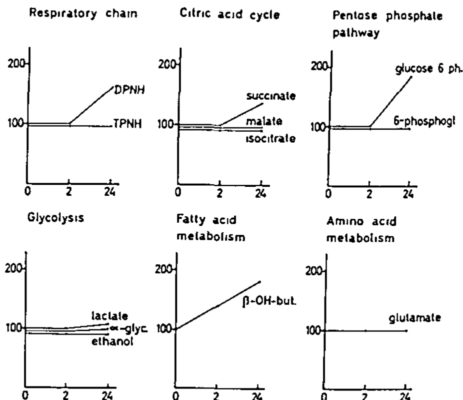


Fig 13

Relative histochemical activity of oxidative enzymes in the straight parts of the proximal tubules after small (0 4 ml/100 g) dose of glycerine given subcutaneously Otherwise as in Fig 11

TABLE 1

Diaphorase Activities in the Kidney Cortex Fractions Measured with DCPIP

	Experiment No	Mitochondria DPNH	Mitochondria TPNH	Microsomes DPNH	Microsomes TPNH	Supernatant DPNH or TPNH
Control	1	1 05	0 17	0 44	0 08	1 32
	2	1 11	0 22	0 57	0 13	1 26
Glycerine 2 hrs	1	0 67	0 15	0 30	0 05	1 22
	2	0 80	0 15	0 35	0 11	1 14
Glycerine 24 hrs	1	0 46	0 08	0 11	0 05	1 55
	2	0 30	0 10	0 19	0 00	1 83

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2	none	13 2	21 5	1 6
	none without phosphate acceptor	0		
3	none	9 8	15 1	1 6
	none without phosphate acceptor	0 7		
4	NaDOC	9 6	16 1	1 7
	NaDOC without phosphate acceptor	0 5		
5	NaDOC	11 7	18 [illegible]	1 5
	NaDOC without phosphate acceptor	0 5		
6	NaDOC	10 5	18 6	1 8
	NaDOC without phosphate acceptor	0 9		

Concerning fractionation procedure measurements and calculations see Materials and Methods

DISCUSSION

Investigations of the reactions of tetrazolium salts with succinate as substrate demonstrated that these electron acceptors do not react with the succinic dehydrogenase but with one or a number of intermediate components in the respiratory chain (*Nachlas et al* 1960, *Wattenberg & Leong* 1960 *Lester & Smith* 1961) If there is also an intermediate between the pyridine nucleotide oxidizing flavoproteins and the tetrazolium salt leakage of this intermediate may cause a diminished histochemical reaction without a decrease of diaphorase activities Recently performed biochemical invest gat on [illegible]

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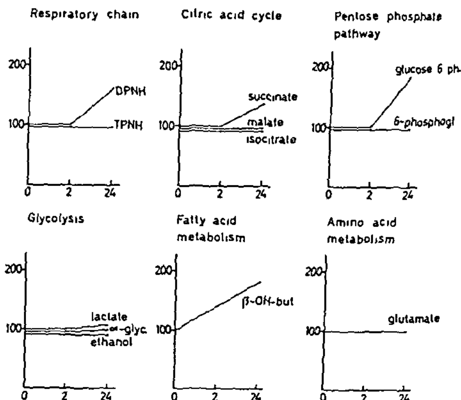


Fig 13

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2 *NaDOC and Small Dose of Glycerine*

Intravenous injection of NaDOC and subcutaneous injection of the small dose of glycerine resulted in increased formazan deposits in the

and that there is a rapid breakdown of the substance within the cytoplasm These processes, as well as the *restoration of membrane systems within the cells are energy requiring* It is questionable whether on the basis of the histochemical findings (*Dallner & Ericsson* 1962) an increased respiration linked to synthesis of ATP offers an explanation of the increase in intensity of the staining reactions for oxidative enzymes after injection of NaDOC and the low dose of glycerine These re[illegible]lts cannot be applied directly to the *in vivo* condition, as the pre-[illegible] one may

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of glycerine did not correspond to a similar change in the activity of β hydroxybutyrate oxidase in isolated mitochondria Tetrazolium salts penetrate some membrane limited structures slowly (*Ackerman* 1960, *Pearse* 1960) and the most probable explanation of the extensive deposition of formazan within the sections is an increased permeability of the *cell and/or mitochondrial membrane caused by* NaDOC and possibly glycerine In the case of NaDOC this explanation is very likely, as this substance is a surface active detergent On the other hand, the effect of glycerine, if any, on the cell membrane remains obscure especially since the intravenous injection did not cause changes in the enzymes measured The possible increase in permeability in the frozen *sections appeared to be a functional change of the cell and/or mitochondrial membrane* but not the sign of actual mitochondrial damage, as was demonstrated by the *full respiratory control* and unchanged P/O ratio in the isolated mitochondria In accordance with the biochemical results no morphological changes in the epithelium were observed by electron microscopy (*Ericsson & Dallner* 1962)

If there had been a real increase in the respiration, stimulated by the absorption of haemoglobin in the tubular cells, it is to be expected that this increased *activity had also* occurred when lyophilized haemoglobin was absorbed after the intravenous *injection Furthermore*, this increase *should have* been more pronounced in the *proximal convoluted tubules*, where the haemoglobin absorption is more extensive, than in the straight *part* The evidence therefore indicates that the increased *formazan* deposition was related to the substances used for the induction *of haemolysis* rather than to the haemoglobin itself

It has been claimed *that the* activity of oxidative enzymes demonstrated with *tetrazolium* salts may *show changes before* structural alterations detectable with the light microscope, have had *time to develop* (*Wachstein* 1955, *Wachstein & Meisel* 1955, *Dallner* 1960) This statement was confirmed in the *light microscopic* studies performed parallel with the histochemical ones on kidneys after injection of the large dose of glycerine, alterations *in many* enzymes occurred several hours before there was light *microscopic evidence of cellular* degeneration and necrosis On the other hand, there seemed to be a *good corre-*

dose of glycerine showed good correlation between the biochemical and histochemical results Thus, the decrease in intensity of the histochemical diaphorase reactions, observed in the kidney after injection of the large dose of glycerine, reflected significant changes of enzyme activities On the other hand, pyridine nucleotide-linked dehydrogenases only react with tetrazolium salts in the presence of DPNH-oxidizing flavoproteins. Therefore, conclusions concerning changes in enzymes such as lactate and DL-β-hydroxybutyrate dehydrogenase must be made with certain reservations

The most evident and pronounced increase in the deposition of formazan in the proximal tubules after NaDOC occurred when DL-β-hydroxybutyrate was used as a substrate for the histochemical reaction Accordingly this substrate was selected for the biochemical control experiment[1] A detailed investigation of the respiration of the mitochondrial fraction with other substrates was not performed, as it could be anticipated that an increase in the respiration in only a small part of the tubules would not be significant, considering the large overall oxygen consumption of the kidney with most of the substrates used The biochemical investigation showed no significant increase in the activity of β-hydroxybutyrate oxidase The findings indicate the necessity of a biochemical control before conclusions concerning quantitative histochemical changes in such enzymes are drawn

The purpose of the investigation was to clarify the effect of haemoglobin on the kidney, with special reference to the rôle of this substance in the tubular changes observed after haemolysis, and it was therefore considered necessary to use homologous haemoglobin In addition, there is some evidence to indicate that there are differences in the way various proteins are handled by the renal tubules (*Oliver et al* 1954), and definite chemical differences have been shown to exist between haemoglobins from various species (*Fruton & Simmonds* 1958)

The intravenous injection of haemoglobin in a dose corresponding to the amount released during haemolysis induced by the large dose of glycerine (*Ericsson* 1964) did not lead to changes in the histochemical reactions of the oxidative enzymes It can thus be concluded, that the tubular necrosis observed after the large dose of glycerine was not primarily related to the effect of haemoglobin

Ultrastructural investigation of the renal changes during haemolysis and haemoglobinuria (*Miller* 1960, *Ericsson* 1962, *Ericsson & Dallner* 1962) have shown that haemoglobin is absorbed by way of pinocytosis

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1 This β hydroxybutyrate oxidase is not the same as β hydroxybutyrate tetrazo [illegible] le terminal respiratory chain [illegible] was assumed that the DPNH- [illegible] the same flavoprotein as that [illegible] furthermore that the rate limiting step of the respiratory chain was at the level of the DPNH dehydrogenase In this way the two enzyme systems are comparable with each other

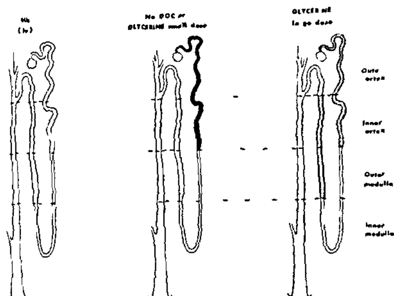


Fig 14

Schematic representation of the changes in the nephron after different experimental procedures Black region indicates increased and shaded decreased activity of oxidative enzymes as revealed by the histochemical technique employed

also indicate that diminished activities of oxidative enzymes precede those of acid phosphatase

The biochemical investigation after the large dose of glycerine showed a pronounced decrease of diaphorase activities both in the mitochondrial and the microsomal fractions DT diaphorase activity which did not show any decrease is not important in cellular respiration and energy production since this enzyme contrary to the particulate diaphorases is not coupled to the respiratory chain and the electron transport linked oxidative phosphorylation (*Danielson & Ernster* 1962)

The results of the experiments are summarized in Fig 14 Contrary to the pure effect of haemoglobinuria haemolysis induced with NaDOC and a small dose of glycerine was followed by increased histochemical reactions of many oxidative enzymes in the proximal part of the nephron After a large dose of glycerine decreased tetrazolium reactions occurred mainly in the proximal tubules and the picture of an "upper nephron nephrosis" rather than a "lower nephron nephrosis" as described by *Campbell* (1961) was attained

SUMMARY

1 The effect of haemolysis and haemoglobinuria on the oxidative enzyme systems in the renal tubules of the rat was investigated with histochemical methods using the tetrazolium technique

lation between the electron microscopic, histochemical and biochemical changes, in that mitochondrial abnormalities in the proximal convoluted tubules (separations of the cristae) occurred 2 hours after the induction of haemolysis, concomitant with decreased activity of some of the mitochondrial enzymes

According to *Cameron & Finckh* (1956) and *Finckh* (1959), the tubular necrosis occurs as a result of the production of a nephrotoxic substance This theory was based on the finding that intraperitoneal injection of glycerine caused kidney damage but no haemoglobinuria We could not confirm these results, as in our hands the intraperitoneal injection of glycerine caused severe haemolysis in many of the animals The above-mentioned authors did not study the amounts of haemoglobin in the plasma If the animals had a slight haemolysis, haptoglobin (an α_2-globin) may have bound all the haemoglobin and no haemoglobinuria would occur (*Jones et al* 1961) The intraperitoneal injection of glycerine caused severe peritonitis, which, in combination with the haemolysis, may offer an explanation of the observed renal changes *There is thus, at present, no positive evidence for the production of a nephrotoxic substance by the subcutaneous injection of glycerine* Taking into consideration the pronounced haemolysis, it would seem more likely that the primary reason for the alterations was hypoxemia and anoxia, possibly with electrolyte imbalance and internal hydronephrosis due to haemoglobin casts acting as contributary factors

This hypothesis is further supported by the results of studies on the effect of ischaemia on the kidney, as after clamping of the renal artery and vein in the rat necrosis occurs within the same parts of the nephron as was seen after the large dose of glycerine, namely, the proximal tubules (*Wachstein & Meisel* 1957)

The early decrease in many of the oxidative enzymes indicates that an important and possibly primary cause of the cell damage was a lack in the production of high energy phosphate The possible rôle of bursting lysosomes, with subsequent release of hydrolytic enzymes into the cytoplasm, as a primary cause of cellular necrosis was pointed out by *de Duve* (1959) In our experimental system it appeared that at least some of the absorbed haemoglobin and acid phosphatase, one of the lysosomal enzymes, were localized in the same organelles (*Ericsson* 1962) This may be explained on the basis of fusion between absorption droplets and lysosomes, as proposed by *Straus* (1963) in the case of horse-radish peroxidase This process, however, seems to be the physiologic reaction of the cell to absorbed substances and does not indicate cellular injury, as it was also observed after the intravenous injection of haemoglobin (*Ericsson* 1964) A histochemical decrease of acid phosphatase activity after the large dose of glycerine was not noted until 8 hours after the injection Biochemical and histochemical studies by *Trump et al* (1963) on the enzymatic changes during "*in vitro necrosis*"

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2 The pure effect of homologous haemoglobin was studied after injection of a large dose of the substance intravenously No changes in oxidative enzyme activities were noticed

3 Haemolysis was *induced by the subcutaneous injection of* glycerine and intravenous injection of NaDOC

4 After a short haemolytic period induced with NaDOC intravenously or a *small* dose of glycerine subcutaneously, increased histochemical reactions occurred in the proximal tubules Biochemical control experiments did not confirm these histochemical findings The increased formazan deposits were thus probably due to the effects of the injected substances, diminishing or removing the permeability barrier of the epithelial cells and/or the mitochondrial membrane

5 The severe haemolysis and haemoglobinuria after a large dose of glycerine subcutaneously caused a rapid and pronounced decrease of the activities of most of the oxidative enzymes in the proximal tubules and led to necrosis of these tubules after 24 hours The enzymatic changes preceded light microscopical evidence of tubular injury by several hours

The primary cause of the damage was probably anoxia, as neither homologous haemoglobin nor glycerine given intravenously had a damaging effect on the renal tubules

6 The effects of haemoglobin, glycerine, and NaDOC on the oxidative enzymes in the epithelial cells of the kidney were discussed as well as *some of the pitfalls of the tetrazolium reaction encountered* in enzyme histochemistry

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THE INFLICTION AND HEALING OF A LARGE STANDARD BURN IN RATS

By

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Received 11 xii 63

In all studies on burns it is difficult to obtain satisfactorily standardized experimental conditions In experiments using small animals and requiring large numbers of animals in each experimental group, followed by statistical analysis of the results obtained, uniform conditions within the individual groups are essential Burn experiments including studies of extensive burns are especially difficult because such burns are not easily inflicted, and here the mortality rises rapidly when the burns exceed a certain degree of severity

In this investigation a standard burn procedure with the following advantages is described (1) the surface area and the degree of severity of the burn can be predetermined, (2) the depth of the burn wound and the borderline between burned and non burned skin is very well-defined, (3) uninjured extremities and body orifices are preserved, (4) complications involved by additional trauma and haemorrhage resulting from autoingestion are eliminated, (5) both small and large burns can be made with reproducible survival rates, and (6) healing occurs under a closed crust, thus minimizing hazards of secondary infection

The method has been tested on a number of rats, which were subjected to burns of different degrees of severity In a group of extensively burned animals, studies with special reference to histopathologic changes during healing of a large burn wound have been made

Method of Inflicting Standard Burns

A series of measurements were made as follows on 20 white male rats of the Wistar strain weighing 200 ± 5 g Each rat was shaved and anaesthetized, upon which a plaster cast was placed over the convexity of its spherically bent back The plaster cupolas thus obtained were found to be almost identical in shape and volume Elliptical openings were cut out of the vertex of six of these cupolas, so that the skin of the

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back, which protruded through the opening, corresponded to approximately 10, 15, 25, 35, 40 and 60 per cent, respectively, of the calculated total body surface area of the animal The total body surface area was calculated according to the formula of *Lee* (1)

$$S = k \cdot w^{n},$$

where S = the total body surface area in cm², k = 12 62 for male rats, w = the weight of the animal in grams and n = ⅔ As regards the larger openings it was found convenient to incorporate some metal wires into the plaster, as a support for the back, so as to prevent the

regulated temperature In this way the mould protected the animal, except for the skin area which was to be scalded, *i e* the elliptical opening (Figs 1 a and b)

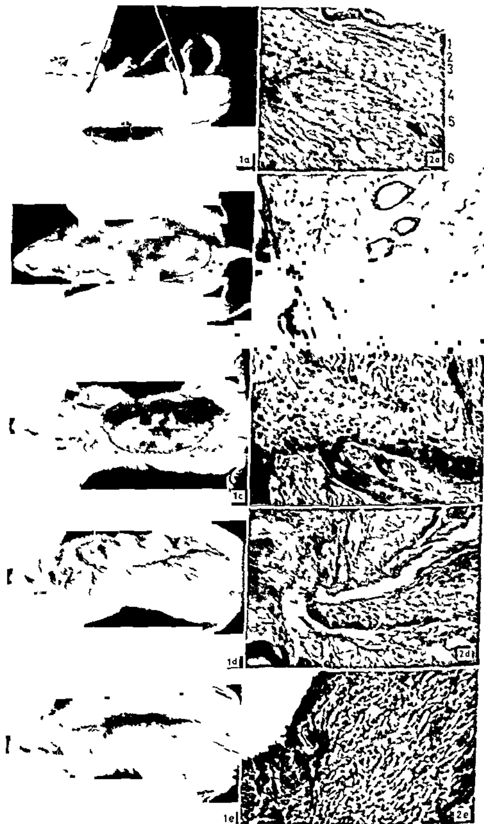
The 20 animals used for the casting of the plaster cupolas were divided into two equal groups Ten animals were used for calculating the standard deviation of the size of the burned surface areas in cases using the 25 per cent mould Skin measurements immediately after application of the burn showed a consistent standard deviation of ±1 5 per cent of the burned surface

The other 10 animals were used for histological estimation of the outer border of the burn wound and its depth, after immersion of the animals for different periods of time in water at +90° C After five seconds' contact with the water, a lesion, corresponding to a second degree burn in man, was found to be produced, while after 20 seconds a third degree burn was produced with coagulation necrosis of the epidermal and the dermal layers of the skin The borderline between burned and non burned skin was very distinct

Figs 1–2

Fig 1 (*a*) A rat in the plaster cast with an elliptical opening equivalent to 25 per cent of the total skin surface area (*b*) The rat 12 hours after scalding (*c*) Three days after application of the burn Haemorrhagic spots and necrotic areas on the burned surface (*d*) Ten days after application of the burn Complete separation of the slough (*e*) 28 days after application showing the burn wound almost healed

Fig 2 Cross sections of skin burned for 20 seconds in water at +90° C (H and E.) (*a*) Immediately after scalding Swelling of the cytoplasm and the nucleus, perinuclear vacuoles, disintegrative necrosis of some nuclei and fluid collected in the stratum lucidum × 320 (1 — S corneum 2 — S lucidum 3 — S granulosum, 4 — S *spinosum*, 5 — S basale 6 — corium) (*b*) 12 hours after scalding "Horns" of the epidermis, oedema and coagulation
...ys after scalding Epithelial
... and ducts × 450 (*d*) Ten
New epithelium is migrating
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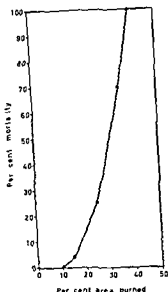


Fig 3
Percentage mortality in relation to area burned

vals and stained with Ehrlich s haematoxylin eosin ...

RESULTS

Mortality Rate

100 per cent mortality was noted in animals with burns extending to more than 40 per cent of the body surface, and 50 per cent mortality in those with burned areas of about 30 per cent (Fig 3)

The following results were obtained by comparisons between the normal control rats in Group 1 and the rats in Group 4 with a third degree burn covering 25 per cent of the total body surface

Body Weights, Water and Food Consumption and Urinary Output

The control animals showed an insignificant loss of weight during the first 24 hours only, which can be ascribed to the decreased consumption of water and food A compensatory increase in weight was evident already on the following day (Fig 4) During the whole observation period, i e three weeks, an average weight increase of approximately 2 0 per cent of the total body weight was recorded each day

The body weight of the burned animals decreased markedly during the first two days following the burn, but subsequently increased from

EXPERIMENTS

In order to test the method described and at the same time to study the histopathological changes to occur in the burn wound in the interval between application of the burn and until complete healing is obtained in severely burned rats a total of 150 white male rats of the Wistar strain were used. The animal material, which is given in its entirety in Table 1, was divided into six different experimental groups.

TABLE 1

Distribution of the Animal Material in the Different Experimental Groups

Experimental group	Percentage of surface area burned	Degree of burn	Number of rats in the different groups
1	Controls		15
2	10	3	15
3	15	3	15
4	25	3	75
5	35	3	15
6	40	3	15
		Total number of rats	150

All animals were carefully observed during a control period of about one month and animals which during this period showed an abnormal weight curve, were in poor condition or had any skin affection, were not included in the experiments. During all the experiments the animals were placed in special, individual cages (2), and regular measurements were made of the body weight, daily urinary output and daily quantity of food and water consumed by the animals. About 24 hours before the application of the burn all hair was removed from the back by razor shaving with soap and water, care being taken not to damage the skin. After shaving the animal was dried and placed in a room at a temperature of +25° C. The burn was produced by immersion into water at +90° C for 20 seconds, animals weighing 200 ± 5 g. All the control animals were shaved and anaesthetized with divinyl ether (3) in the same way as the experimental animals but were lowered into a water bath at body temperature.

Water was given *ad libitum* to all the animals and the food given consisted of a standard diet of the following composition: carbohydrate 51 per cent, fat 32 per cent and protein 17 per cent. The diet was supplemented with McCollum's salt mixture 1.8% and vitamins. No local or general treatment was given. No dressing was used.

Haematocrit determinations were carried out in an IEC international microcapillary centrifuge (model MB) on the smallest possible quantity of blood in order to avoid anaemia. No correction was made for trapped plasma.

To obtain some idea of the relation between the mortality rate and the extent of the burn 15 animals from each of the experimental groups were used for survival studies alone. The remainder of the animals in Group 4 were used for wound studies.

The percentage quantity of "free water" was calculated in different skin areas. For this purpose tissue pieces of approximately equal size were dehydrated to constant weight at a temperature of +105° C in a thermostat containing a water absorbing medium (silica gel), i.e. for three or four days. The term "free water" is here used to denote the water removed during this dehydration process. Although there is no sharp borderline between "free" and "bound" water it is nevertheless possible to compare the "free" water values obtained on different occasions from each skin piece, since they were dehydrated in the same way. Scabs were not included in the samples.

In order to obtain a clear distinction between the process of epithelialization and the contraction of the wound, the edge of the burned surface was tattooed with India ink immediately after application of the burn, the tattoo points marking the advancing edge of the wound during contraction. Every day after scalding the wound was traced, the border of the tattoo points being marked on translucent paper

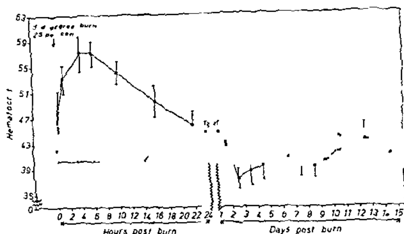


Fig 5

Haematocrit values in the rats in Gr up 4 at different times after burning (25 per cent third degree) The vertical lines indicate the standard deviation

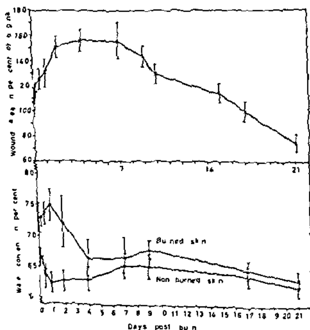


Fig 6

W und area as percentage of original wound area and water content in burned and non burned skin in the rats in Group 4 (25 per cent third degree burn) The vertical lines indicate the standard deviation

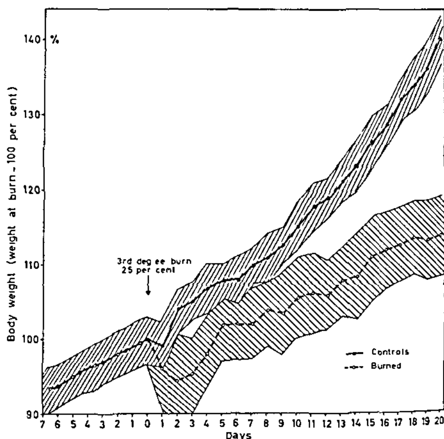


Fig 4

Body weight as percentage of original body weight in control and burned animals (third degree burn, covering 25 per cent of the body surface)

the third day and onwards (Fig 4) The average weight increase during the three weeks subsequent to the burn was only 0 7 per cent of the total body weight per day

The food consumption also varied in the two groups The control animals ate, on an average, 14 1 g/100 g body weight in 24 hr of the standard diet, while the burned animals consumed, on an average, only 4 2 g 100 g body weight in 24 hr during the first two days following the burns Subsequently their appetite gradually increased

The burned rats drank very little during the first 24 hours after scalding and periods of anuria were often noted

Haematocrit Determinations and Calculation of the Water Content in Burned and Non-Burned Skin

The mean value and the standard deviation of the haematocrits of the animals before the burn were 42 ± 1 8 per cent

A substantial rise in the haematocrit values during the first 24 hours after application of the burn was followed by a decrease which did not reach normal values until 10–12 days later (Fig 5) There was a

across the wounds beneath the overlying slough or crust separating the dead tissue were found (Fig 1 d and 2 d) New capillaries migrated across the wound upwards from its base

Already within the second week *after burning* the crust was dry and separated and the size of the wound area began to decrease more rapidly due to increasing contraction At the same time granulation tissue was detectable with capillary buds, fibroblasts, and leucocytes About four weeks after application of the burn an epithelial surface was reformed (Figs 1 e and 2 e)

In some rats the development of granulation tissue was poor, usually due to severe local infection If so, the remaining dermis and subcutaneous fat were infiltrated with numerous leucocytes and abscesses (Fig 7 a, b), in these cases dermal cyst formations (Fig 7 c) and thrombotic vessels (Fig 7 d) were sometimes seen

About five weeks after the trauma the wound had healed completely, new epithelium covering entirely the granulating surface Initially the stratum papillare failed to develop and the elastic tissue was poor About two months after application of the burn the hair follicles and the glands were seen to have normalized and the collagen fibres of the connective tissue had increased in number

DISCUSSION

Several different methods have been described which try to produce burns on experimental animals, *e g* branding-irons, flames, steam, hot water, etc (4, 5, 6, 7, 8, 9, 10, 11) Among these methods the branding-iron seems to be most adequate fulfilling most of the requirements set up in the introduction, when small burns are to be produced In order to produce a large burn, however, a number of "brandings" must be performed This process is rather time-consuming and it is hardly possible to fix the exact time at which the burn was inflicted Besides, the total surface area of these "brandings" is not synonymous with the total burn produced, as the bridges of skin between "brandings" also are more or less damaged Moreover, the depth of the burn is influenced by the degree of pressure on the skin by the branding iron, and it may be difficult to keep this pressure constant, unless the surface is very small The method by which to inflict standard burns developed in this investigation has none of these disadvantages and seems to be especially useful for the production of large burns

The initial increase of the haematocrit coincided with a rapid increase of the water content in the burned tissue and a slower increase of the wound surface area At the same time the water content in non-burned skin decreased These results indicate that some of the water released from the blood stream into the burned tissue is compensated by withdrawing water from non burned tissue During the second and third days after scalding, when the reabsorption of water from the burned

striking correlation between the haematocrit values and the general condition of the animals

A rapid initial increase in the water content in burned skin was found As no external secretion from the wound was visible during the first two days, the water quantities measured may be regarded as a good gauge of the amount of oedema In the non-burned skin area a marked *decrease of the water content was found, which became more and more* pronounced as shock developed About a week after scalding the water content, both in burned and in non-burned skin, had returned to normal (Fig 6)

The wound area increased in size during the first two days after scalding later to show a fairly constant size From the end of the first week and onwards a gradual approximation of the wound edges was observed, reducing the wound area by about 5 6 per cent per day About two weeks after application the wound was of the same size as immediately after application, and had healed another three weeks later

Histopathologic Changes During Healing of the Burn Wound

Immediately after scalding the burn wound rose above the surrounding skin, it became pale, cyanotic and spongy, and lost its elasticity The microscopic picture showed swelling of the cytoplasm and nucleus of tissue cells with some loss of cytoplasmic basophilia, a more uniform staining of the nucleus and the appearance of clear perinuclear vacuoles (Fig 2 a)

During the first few hours after burning a rapid increase of wound oedema was found (increasing water content and wound area) The stratum lucidum was destroyed and the cells of the stratum granulosum and germinativum showed large perinuclear spaces The stratum corneum was *loosened into its component layers, showing epidermal* "horns" and in the dermis a coagulation necrosis was found (Fig 2 b) with rupture of the fat cells of the sebaceous glands, cytoplasmic eosinophilia and lysis of the cell nuclei of the hair follicles and sweat ducts The collagen fibres were swollen, fused into solid masses and lost their affinity for acid dyes Dilated and stased capillaries, plugged with closely packed red cells, were common The subcutaneous fat was destroyed and replaced by oedema containing red and white blood cells

About three days after burning and onwards the wound *oedema* decreased slowly and haemorrhagic spots and necrotic areas appeared on the burned surface (Fig 1 c) The slough was formed and demarcated by leucocytes in the deeper part of the dermis and subcutaneous tissue A rapid epithelial multiplication and migration started (often within 24 hours after application) uppermost in the surviving parts of the viable hair follicles and ducts and from the edge of the wound before any new connective tissue was detectable (Fig 2 c) At the end of the first week after the trauma *epithelial cell formations migrating*

area exceeded the oedema formation, the haematocrit fell to subnormal values This fall of the haematocrit is also due to the disappearance of red cells from the active circulation, which has been shown to occur also in patients with burns (12, 13) as in burned animals (8, 14) While the content of water in the burned tissue decreased, the wound showed constant size Not until about a week after application of the burn a more pronounced decrease of the wound was manifest, caused by contraction

The histologic examination of the burned tissue showed that the repair of the wound started with autolysis and separation of the dead tissue This seemed to be caused by epithelial cell formations migrating initially across the wound beneath the overlying slough During the second week the contraction of the wound became more obvious and granulation tissue was detectable The hyperplasia of epidermal cells usually started already within the first few days after scalding Similar findings in burned dogs have been reported by *Price et al* (15) The onset of contraction of the wound as well as of formation of granulation tissue, was delayed as compared with that in a full thickness excised wound (16, 17), probably due to the slough As soon as the slough had separated from the edge of the wound, a reduction of the wound area by about 5 6 per cent per day was found, *i e* results similar to those reported by *Blair et al* (17)

In some animals the healing was delayed by wound infection It is also possible that the healing of a burn wound could be delayed by the disappearance of red cells from the active circulation mentioned above, as well as by the low serum protein concentration following scalding, which has been discussed earlier (18)

SUMMARY

The technique of inflicting standard burns on rats is discussed in detail The advantages of the burn procedure in the present investigation are summarized in the introduction

In a group of extensively burned rats (25 per cent, third degree burn) the histopathologic changes of the wound to occur in the interval between the scalding process and complete healing have been investigated

It was found that some of the water released from the blood stream into the burned tissue during the first few days was compensated by water withdrawn from non burned tissue The repair of the wound started with epithelial cell formations migrating across the wound beneath the overlying slough or crust separating the dead tissue As soon as the slough had separated from the edge of the wound, the contraction was accelerated and granulation tissue was detectable

Both the formation of granulation tissue and the onset of the contraction were delayed as compared with the behaviour of full thickness

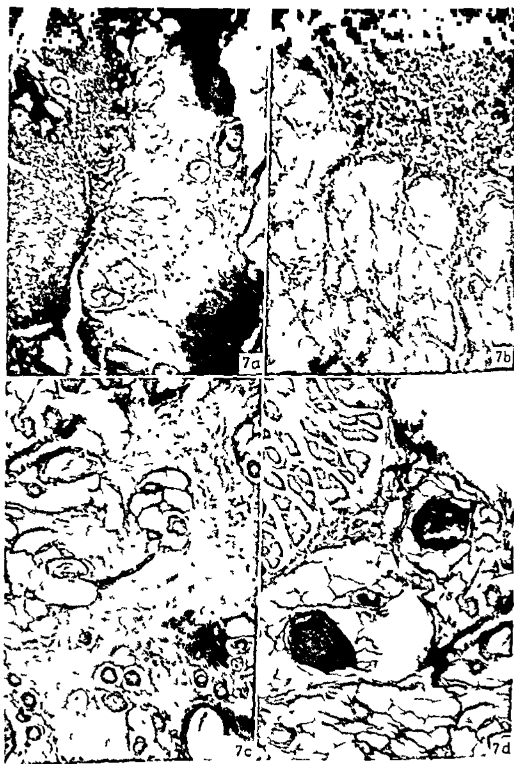


Fig 7

Cross sections of skin burned for 20 seconds in water at + 90° C (H and E) (*a*) 26 days after application of the burn Delayed healing due to severe local infection × 120 (*b*) 28 days after application Massive infiltration of inflammatory cells in the deep tissue × 200 (*c*) 30 days after application Dermal cyst formation × 200 (*d*) 30 days after application Thrombotic large vessels in the subcutaneous tissue × 320

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Department of Human Anatomy University of Copenhagen Nørre Allé 63 Laboratory of Electron Microscopy Laboratory of Cyto and Histochemistry and Laboratory of Experimental Embryology

ON THE NATURE OF THE MECONIUM CORPUSCLES IN HUMAN FOETAL INTESTINAL EPITHELIUM

1 *Electron Microscopic Studies*

By

F BIERRING, H ANDERSEN, J EGEBERG, F BRO RASMUSSEN and M MATTHIESSEN

Received 17 xii 63

In 1905 *Schmidt* reported that the epithelial cells of the villi in the small intestine of human foetuses contain large spherical, homogeneous or granular, yellowish inclusions *Schmidt*'s original observation was repeated by *Parat* (1922), who in accordance with Schmidt found that the inclusions can be demonstrated from the 14th week, and that they are largest and appear in greatest numbers in the distal part of the small intestine The number and size of the inclusions increase with increasing foetal age, so that, towards the end of the 6th month, they almost completely fill the epithelial cells of the human ileum Subsequently, they gradually decrease, and at birth they only occur in the epithelial cells at the top of the villi where, for that matter, their number has all the time been greater than in the cells near the base of the villi They are never found in the epithelial cells of the crypts

Schmidt (1905) further found that inclusions of similar appearance occur in the epithelium of the small intestine in newborn dogs, an observation which previously had been made by *Heidenhain* (1888), and *Parat* (1924) described the presence of the inclusions in new born mice, guinea pigs cats, and sheep In these animals the inclusions did not occur in the foetal intestine

Schmidt (1905) assumed that the inclusions consist of absorbed material including bile pigment, from meconium and amniotic fluid This concept however does not fit in with *Tobeck*s (1925) observation of similar inclusions in the intestinal epithelium distal to an atresia The inclusions resemble the yellowish green corpuscles found in the meconium first described by *Tardieu & Robin* (1857) Owing to this resemblance they are usually called meconium corpuscles, although their nature, their name notwithstanding, has not been definitely established

excised wounds in rats reported in the literature Wound infection delayed the wound healing further

About five weeks after scalding the wound was completely healed

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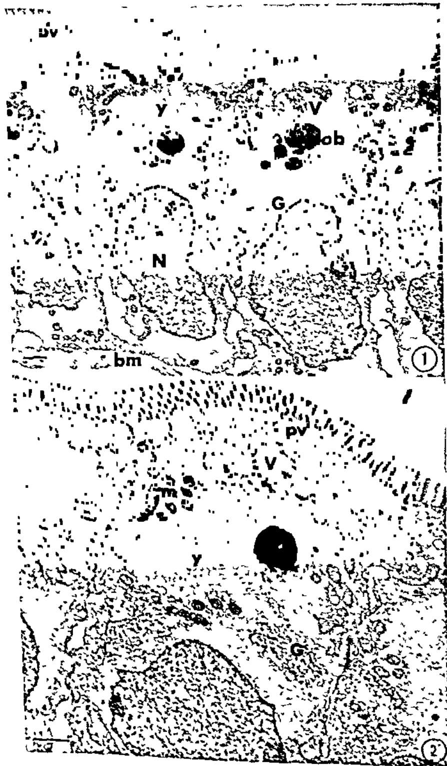
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In the present paper some features of the ultrastructure of the meconium corpuscles are described in an attempt to classify them within the number of known subcellular elements

MATERIAL AND METHODS

The material of the investigation consists of intestinal mucosa from 5 normal human foetuses, delivered by hysterotomy in cases of legal abortion carried out in connection with sterilization procedures The crown rump lengths of the foetuses were 113 mm 125 mm, 150 mm, 151 mm, and 152 mm, corresponding to a fertilisation age of 14, 15 and 17 weeks

Immediately after removal from the uterus the abdomens of the foetuses were opened, and small segments were excised from the middle part of the small intestine The specimens were fixed in a 1 per cent solution of OsO_4 in veronal acetate buffer (*Palade* 1952) at 4° C for 1 hour, dehydrated in alcohol, and embedded in methacrylate Further, adjoining segments of the small intestine were fixed in 10 per cent neutral formalin and embedded in paraffin

Sections of the methacrylate embedded tissue were cut on an LKB ultratome 1 2 µ

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eosin for ordinary light microscope study

OBSERVATIONS AND DISCUSSION

The epithelial cells of the villi in all the foetuses studied contained inclusions, the morphology and topography of which were in close conformity with the descriptions given by *Schmidt* (1905) and *Parat* (1922) The inclusions, which are always located in the luminal half of the cells within supranuclear light areas of the cytoplasm, are yellowish or yellowish-green in sections stained with haemotoxylin-eosin, and dark by phase contrast microscopy Their ultrastructural counterpart (Fig 1)

Key to Abbreviations Used in all Figures

bm = basement membrane		pv = pinocytotic vesicles	
ob = osmiophilic bodies		i = intercellular space	
G = Golgi apparatus		N = nucleus	
y = glycogen		V = vacuoles	
m = mitochondria			

Unless otherwise indicated the magnification mark appearing on the electron micrographs indicates 1 µ

Figs 1 2

Fig 1 Low power electron micrograph showing epithelial cells on a small intestinal villus from a human foetus of 150 mm crown rump length Large amounts of stored glycogen occupy the cytoplasm on all sides of the nuclei Numerous pinocytotic vesicles are seen in the glycogen free cytoplasmic zone at the luminal end of the cells Large vacuoles are situated deeper in the cells The osmiophilic bodies which correspond to the meconium corpuscles of light microscopy are located in the central part of the supranuclear glycogen containing cytoplasmic area

Fig 2 Pinocytotic vesicles vacuoles and an osmiophilic body in the supranuclear part of a villus epithelial cell The Golgi apparatus is found at the level of the apical pole of the nucleus


pv
v
y
ob
i
3
pv
v
0.5
4

are polymorphous, osmiophilic, electron-dense bodies of a round, oval, or more irregular shape. The identity appears from the localization of the osmiophilic bodies as well as from their size, which in most cases greatly exceeds the resolution of the light microscope, and the identity could be further established by phase contrast microscopy of thicker sections and electron microscopy of thinner sections of the same cells

The epithelium of the villi of human foetuses in the 4th and 5th month mainly consists of about 13 μ high and 5 μ wide cells, which show a considerable uniformity as regards outer form and inner structure (Fig. 1) A conspicuous feature in the morphology of the cells is their large content of stored glycogen, which to the exclusion of almost all other components of the cytoplasmic matrix occupies the cytoplasm on all sides of the nucleus Mitochondria, endoplasmic reticulum, and the Golgi apparatus (Fig 2), which surrounds the apical pole of the nucleus, are confined to a narrow, peripheral, glycogen-free, cytoplasmic area The glycogen-free area is widest at the luminal end of the cell, where, in addition to mitochondria and endoplasmic reticulum, it contains numerous small, clear, membrane-bounded vesicles The vesicles apparently represent pinched-off invaginations of the plasma membrane between the short, plump microvilli lining the luminal surface of the cell (Figs 2 and 3) They are round, ovoid, or pear-shaped, and, often arranged in rows, they stretch towards the interior of the cell, where they fuse with large membrane-bounded vacuoles (Fig 4) The vacuoles, which presumably arise from the fusion of smaller vesicles are situated at the margin of the glycogen-containing zone Smaller vacuoles appear almost empty, whereas the larger ones have a diffuse, floccular content, often with aggregations of amorphous material of a low electron density The findings suggest that by pinocytosis the epithelial cells take up dissolved substances, which are conveyed in pinocytotic vesicles to larger vacuoles, where they are condensed and precipitated

The osmiophilic bodies which correspond to the light-microscopically visible inclusions are found basal to the vacuoles, and always in the central part of the large supranuclear, glycogen-rich cytoplasmic area The large numbers in which they are present, are evident from the fact that almost all cells, even in the ultrathin sections, contain numerous bodies Their size varies from approximately 0 3 μ to 2 μ as their largest diameter, and in addition to round and oval profiles, there are more irregular, hand mirror-like (Fig 5) or dumb bell-like (Fig 6) forms,

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Figs 3–4

Fig 3 Pinocytotic vesicles in the luminal glycogen-free cytoplasmic zone The figure illustrates that the vesicles probably represent pinched off invaginations of the luminal plasma membrane An osmiophilic body to the right in the figure is at some distance partly surrounded by two membranes

Fig 4 Higher magnification micrograph showing pinocytotic vesicles a vacuole and osmiophilic bodies in the supranuclear area of a villus epithelial cell A pinocytotic vesicle apparently fuses with the vacuole


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5

0,5
6

which suggest that smaller bodies can be taken up into larger bodies Based on their inner structure they may be divided into 2 types

The most frequent type of bodies is completely filled by a homogeneous, dense matrix containing small, dark particles, and is surrounded by a single or a double membrane. The particles are either diffusely distributed (Fig 3), or they are mainly located in certain areas of the matrix (Figs. 4, 5, 6, 7 and 9) In the latter case they are seen as irregular, filamentous formations in the interior of the matrix (Fig 6, upper body), or as an almost continuous layer close to the inner surface of the outer limiting membrane (Fig 6, lower bodies), an arrangement which in many, and perhaps in all bodies, is responsible for the apparently double character of the limiting membrane Certain bodies of this type contain small, open or closed systems of short parallel lamellae, which are always found in the peripheral part of the matrix (Figs 4, 5, 9, and 10), presumably indicating a sparse content of lipoprotein in this part of the matrix Other bodies of this type have an interior of high electron density (Figs 2 and 8) owing to a content of coarser masses of strongly osmiophilic material These bodies have a single, often poorly defined surface membrane

The other type of inclusions, according to their morphology, represents intermediate forms between the bodies just described and the more luminally located vacuoles (Figs 1, 4, 5, 8, 9, and 11). They are usually smaller, and their content is more polymorphous Some contain sparse amorphous masses and small vesicular elements (Fig 11), others have a content of denser material, in which numerous vacuoles are seen (Fig 9) Lamellar systems are not seen in these bodies

A peculiar type of inclusions is finally seen in Figs 6 and 7 They bear a striking resemblance to the "vacuolated bodies" which *Miller* (1960) found in the cells of the convoluted tubules in mice during protein absorption Their matrix, which is always vacuolated, is excentrically placed and almost semilunar in shape and from the ends of the crescent extensions of their surface membrane stretch out around neighbouring bodies, which they completely or partly surround Ap-

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Figs 5–6

Fig 5 The two large bodies one of which is hand mirror shaped represent the most frequent occurring type of osmiophilic bodies They are surrounded by a single or a double membrane and they are completely filled by a dense matrix containing small dark particles Small systems of short parallel lamellae are seen in the peripheral part of the matrix of one of the bodies (arrows)

Fig 6 A variety of osmiophilic bodies in the supranuclear area of a villus epithelial cell The small dark particles in the matrix of the upper body are arranged in filamentous formations In the lower bodies they form an almost continuous layer close to the inner surface of the outer limiting membrane Membrane extensions from a crescent shaped vacuolated body completely surround another osmiophilic body and further partly surround other osmiophilic bodies Another body is at some distance completely surrounded by two membranes

0.5

7





parently, the membrane from the crescent-shaped body in Fig 6 has under duplication and subsequent fusion of the two duplicates completely surrounded another body, and a new duplicature of the outermost membrane of the two thus formed almost surrounds other bodies Bodies of this appearance hardly constitute a special type, their presence is more likely a further indication that smaller osmiophilic bodies can be taken up into larger bodies, and their appearance suggests that this is brought about by a "phagocytosis at the organelle level" The double membranes, which at some distance completely surround one of the bodies in Fig 6, and which partly surround a body in Fig 3, probably indicate that these bodies are in the process of being taken up into other bodies by means of extensions from the surface membranes of these bodies.

Osmiophilic bodies were never observed in the epithelial cells of the crypts, neither did these cells contain pinocytotic vesicles or vacuoles

The observations reported suggest that vacuoles, formed by confluent pinocytotic vesicles, under progressive condensation of their content are transformed into osmiophilic bodies The osmiophilic bodies thus are the last morphological stage in a series of events associated with the uptake in the cell, by pinocytosis, of material from the intestinal lumen The correctness of this concept is corroborated by the fact that exactly the same morphological stages of incorporation have been described in numerous other animal cells, which take up macromolecular material or insoluble particles by pinocytosis and phagocytosis, and in several investigations (*Karrer* 1958, 1960, *Farquhar & Palade* 1960, 1962, *Miller* 1960, *Easton, Goldberg & Green* 1962) it has even been possible by means of tracer-particles to follow the successive incorporation of the particles into a system of vesicles, vacuoles and osmiophilic bodies, and thus to establish the functional continuity of this system

The electron microscopic study thus has confirmed *Schmidt*'s (1905) concept of the nature of the meconium corpuscles, having shown that they are a product of condensed and segregated material ingested by the cell

In recent years it has been repeatedly shown (*Bennett* 1956, *Straus* 1959, *de Duve* 1959, 1963, *Novikoff* 1959, 1961, *Farquhar & Palade* 1960, *Essner* 1960, *Essner & Novikoff* 1961) that cytoplasmic bodies containing phagocytosed or pinocytosed material belong to the group of sub-

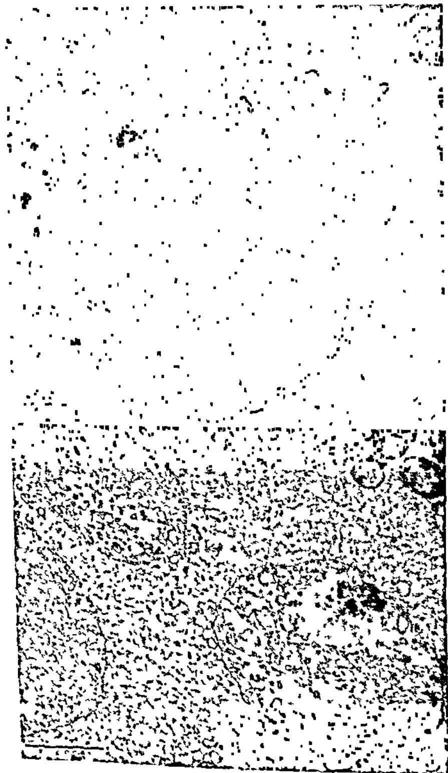
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Figs 7–8–9

Fig 7 Several bodies of another villus epithelial cell The upper body shows short lamellar systems in the peripheral part of the matrix (arrow) One of the vacuolated bodies is crescent shaped and its membrane surrounds another body

Fig 8 An osmiophilic body containing coarser masses of osmiophilic material

Fig 9 A small body with a vacuolated matrix and a larger compact body Short lamellar systems at arrow



cellular elements designated as lysosomes, and are characterized by a high content of acid, hydrolytic enzymes The occasional occurrence in the present investigation of lamellar systems suggests that the content in some of the osmiophilic bodies undergoes at least a partial digestion It is reasonable therefore to assume that the osmiophilic bodies belong to the lysosome group, but the final confirmation of their lysosome character must await the results of cytochemical investigations which are in progress

Cytoplasmic bodies containing other cytoplasmic components which are in a state of more or less advanced disintegration, have most recently been described by *Ashford & Porter* (1962) and *Novikoff & Essner* (1962) in liver cells, and by *Moe & Behnke* (1962) in the small intestinal epithelium of new-born rats These bodies are generally also interpreted as being lysosomes, and it is assumed that they are engaged in a process of isolated cytoplasmic degeneration, but, so far, it is unknown how *organelle containing cytoplasmic areas gain access to the interior* of the lysosomes Bodies of this type have not been observed in the present investigation, but the observations described above make it reasonable to assume that the explanation of the mode of formation of circumscript cytolytic pockets might be sought in a mechanism similar to that which apparently is responsible for the uptake of osmiophilic bodies into other bodies

SUMMARY

The meconium corpuscles, which occur in the epithelial cells of the villi in the small intestine of human foetuses from the 4th to the 7th month, consist of condensed material ingested by the cells by pinocytosis Apparently, the ingested material undergoes partial digestion in the meconium corpuscles, which must therefore be presumed to be lysosomes

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Figs 10 11

Fig 10 Lamellar systems in the peripheral part of the matrix of a large osmiophilic body

Fig 11 Bodies having a polymorphous content of amorphous material and small vesicular elements Bodies of this type as well as the bodies having a vacuolated matrix probably represent intermediate forms between the more luminally located vacuoles and the large compact osmiophilic bodies

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Department

Nørre Allé 63

ON THE NATURE OF THE MECONIUM CORPUSCLES IN HUMAN FOETAL INTESTINAL EPITHELIUM

2 A Cytochemical Study

By

HELGE ANDERSEN, FRANZ BIERRING, MARTIN MATTHIESSEN, JØRN EGEBERG and FREDE BRO RASMUSSEN

Received 17 xii 63

Relatively little seems to be known about the resorptive and metabolic status of the human foetal intestine As summarized by *Patzelt* (1936) previous authors have described cytoplasmic particles in the intestinal epithelium and have assumed that these particles were absorbed meconium for which reason they have been given the name of meconium corpuscles Similar findings have been described by *Bloom & Fawcett* (1962) The corpuscles in question are found in the foetal intestine from the 4th to the 7th month whereupon they disappear

Since *de Duve* (1959) described the lysosomes as a new cytoplasmic group of particles much attention has been focused on these particles, which are grouped with the organelle fraction of the cells

According to de Duve the lysosomes are of varying size averaging 0 4 microns and are surrounded by a lipo protein membrane According to the most recent investigations they contain at least 10 different hydrolytic enzymes which all exhibit optimum activity at acid pH (*Barka & Anderson* 1963) Of these the acid phosphatases are, histochemically, the most easily demonstrable fractions and according to *Novikoff* 1957) the presence of this enzyme is the most reliable cytochemical characteristic of lysosomes

The lysosomes appear to be evident rather frequently and have been described in embryonic as well as in adult types of cells, in the same way they are found in cells of epithelial as well as of mesenchymal origin e g in liver cells where they were first isolated by differential centrifugation and in reticulo endothelial cells (*Straus* 1959), where they are identical with phagosomes

Through collateral electron microscopic studies (*Bierring et al* 1963) lysosome like particles have been found in the human foetal intestinal epithelial cells and the aim of the present study has therefore been to

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Amino Acids

1 Bennett s 1 (4 chloromercuriphenylazo) 2 naphthol (Mercury orange) for SH groups (*Bennett & Watts* 1958) The method is used in the *Mescon & Flesch* modification cited by *Lillie* (1954) with 80 per cent ethanol as the solvent Staining time

(*Pearse* 1960)
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Nucleic Acids

1 The chromalun gallocyanine method for ribonucleic acid (*Einarson* 1951 *Pak Lenberg* 1962)

Phospholipids

1 The copper phtalocyanine method for phospholipids (*Kluver & Barrera* cited by *Pearse* 1960)
2 Menschik s Nile-blue method for phospholipids (*Pearse* 1960)

Lipofuscins

1 Gomori s chromalun haematoxylin method for lipofuscins (*Pearse* 1960)
2 Lillie s alternative Nile blue method for lipofuscins (*Pearse* 1960)
3 *Long Ziehl Neelsen method for the acid fastness of lipofuscins (Pearse 1960)*

Choline Containing Lipids

1 The phosphomolybdic acid method (*Pearse* 1960)

Glycogen and Glyco Lipoprotein

1 The periodic acid Schiff method (*Pearse* 1960) For the demonstration of glycogen prior to the periodic acid the sections were coated with a thin film of 1 per cent celloidine in *equal parts* of ether and ethanol as the periodic acid might otherwise cause complete loss of glycogen—particularly in embryonic cells (*Andersen* 1963 a)

Mucopolysaccharides

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verify these findings from a cytochemical point of view, as well as to map out the chemical processes which take place in these lysosomes

MATERIAL

The material comprises 6 human foetuses removed by Caesarean section in connection with a well-founded interruption of pregnancy Based on crown rump lengths the material is divided up as follows 53 mm, 62 mm, 119 mm, 136 mm 151 mm and 172 mm The foetuses were measured prior to fixation in a supine position unnecessary stretching having been avoided Material from the jejunum as well as from the ileum has been used

METHODS

Fixation This was done within the first 15 min after removal of the foetuses from the uterine cavity Ice cold fixatives were used and the fixation was performed at 0—4° C

The following fixatives were used I 4 per cent formaldehyde (10 per cent formalin) buffered to pH = 6 8–7 0 (*Pearse* 1960) II Lillie s ethanol formalin acetic acid (*Lillie* 1954) Experiments soon revealed that the latter fixative resulted in a complete loss of the cytoplasmic particles in the epithelial cells It was therefore investigated which one of the three components of the fixative was responsible for the above loss, and control experiments using 96 per cent ethanol as a fixative showed that the effect was caused by ethanol

Fixation in formalin was used partly as a fixation of enzymes, partly as a fixation of phospholipids, lipo protein, and protein components For fixation of the enzymes different times of fixation were used, and it was found that satisfactory preservation of alkaline phosphatase and lactic dehydrogenase was obtained when unfixed sections or sections fixed for 15 min were used which time of fixation also gave a satisfactory preservation of amino peptidase For the demonstration of succinic dehydrogenase unfixed sections were used As regards fixation of acid phosphatases and unspecific carboxyl esterase, good results were achieved if the time of fixation covered 3–4 hours whereas no reaction was seen in respect of acid phosphatases when unfixed sections were used which latter observation is in good agreement with *Burstone* (1958) according to whom most hydrolytic enzymes require a certain fixation in order to avoid complete diffusion in the aqueous media of the incubating medium For the fixation of phospholipids lipo protein and components of protein a time of fixation of 18 hours was used

Lillie s fixative and a time of fixation of 18 hours was used for the fixation of glycogen mucopolysaccharides and ribonucleic protein

Embedding and sectioning The preparations for the demonstration of enzymes were made by transferring tissue which had undergone fixation to two shifts of arabic gum sucrose (ice cold) This solution preserves the enzymic activity and facilitates the subsequent cutting on a freezing microtome on which the tissue can be embedded in a small pyramid of the above solution The thickness of the sections was 10 microns ,

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of the other components mentioned

With a special view to the preparation of unfixed frozen sections for the demonstration of succinic dehydrogenase it was endeavoured to keep the block and the knife at a constant temperature in order to avoid thermal damage to the mitochondria with subsequent large deposits of formazan in dilated mitochondria

Histochemical Reactions

Enzymes

1 Modified coupling azo dye method for alkaline phosphatase with a naphthyl phosphate as a substrate and Fast Red TR as a diazonium salt (*Pearse* 1960) pH = 9 2 Time of incubation 2 min 5 min and 10 min

2 Naphthol AS BI phosphate method for acid phosphatase (*Burstone* 1958) with

Red Violet LB as a diazonium salt and at pH = 5 4 Time of incubation 7 min, 9 min 10 min and 1[illegible]
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2 Millon reaction for tyrosine (modified by *Andersen* 1963)

3 Baker s modification of the Sakaguchi reaction for arginine (*Pearse* 1960)

4 Yasuma and Itchikawa s ninhydrin *Schiff method for protein bound* NH_2 (*Pearse* 1960)

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Mucopolysaccharides

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Fixation in formalin was used partly as a fixation of enzymes partly as a fixation of phospholipids lipo protein and protein components For fixation of the enzymes different times of fixation were used and it was found that satisfactory preservation of alkaline phosphatase and lactic dehydrogenase was obtained when unfixed sections or sections fixed for 15 min were used which time of fixation also gave a satisfactory preservation of amino peptidase For the demonstration of succinic dehydrogenase unfixed sections were used As regards fixation of acid phosphatases and unspecific carboxyl esterase good results were achieved if the time of fixation covered 3 4 hours whereas no reaction was seen in respect of acid phosphatases when unfixed sections were used which latter observation is in good agreement with *Burstone* (1958) according to whom most hydrolytic enzymes require a certain fixation in order to avoid complete diffusion in the aqueous media of the incubating medium For the fixation of phospholipids lipo protein and components of protein a time of fixation of 18 hours was used

Lillie s fixative and a time of fixation of 18 hours was used for the fixation of glycogen mucopolysaccharides and ribonucleic protein

Embedding and sectioning The preparations for the demonstration of enzymes were made by transferring tissue which had undergone fixation to two shifts of arabic gum sucrose (ice cold) This solution preserves the enzymic activity and facilitates the subsequent cutting on a freezing microtome on which the tissue can be embedded in a small pyramid of the above solution The thickness of the sections was 10 microns

For the demonstration of cholin containing lipids frozen sections were also used as was the case also with a few reactions for phospholipids For the demonstration of the other components mentioned ordinary paraffin embedded sections were used

With a special view to the preparation of unfixed frozen sections for the demonstration of succinic dehydrogenase it was endeavoured to keep the block and the knife at a constant temperature in order to avoid thermal damage to the mitochondria with subsequent large deposits of formazan in dilated mitochondria

Histochemical Reactions

Enzymes

1 Modified coupling azo dye method for alkaline phosphatase with α naphthyl phosphate as a substrate and Fast Red TR as a diazonium salt (*Pearse* 1960) pH = 9 2 Time of incubation 2 min 5 min and 10 min

2 Naphthol AS BI phosphate method for acid phosphatase (*Burstone* 1958) with

of incubation 7 min 9

Red RC as a capture diazonium reagent

succinate as a substrate and Nitro BT as a hydrogen acceptor time of in u
at pH = 7 4 and at 37° C 7 min 9 min 10 min 12 min 13 min 14 min and 15 min

Amino Acids

1 Bennett s 1 (4 chloromercuriphenylazo) 2 naphthol (Mercury orange) for SH groups (*Bennett & Watts* 1958) The method is used in the *Mescon & Flesch* modification cited by *Lillie* (1954) with 80 per cent ethanol as the solvent Staining time 6 hours

2 Millon reaction for tyrosine (modified by *Andersen* 1963)

3 Baker s modification of the Sakaguchi reaction for arginine (*Pearse* 1960)

4 Yasuma and Ichikawa s *ninhydrin Schiff method for* protein bound NH₂ (*Pearse* 1960)

Nucleic Acids

1 The chromalum gallocyanine method for ribonucleic acid (*Einarson* 1951 *Pakkenberg* 1962)

(*Klüver & Barrera* cited

rse 1960)

Lipofuscins

1 Gomori s chromalum haematoxylin method for lipofuscins (*Pearse* 1960)

2 Lillie s alternative Nile blue method for lipofuscins (*Pearse* 1960)

3 Long Ziehl Neelsen method for the acid fastness of lipofuscins (*Pearse* 1960)

Cholin Containing Lipids

1 The phosphomolybdic acid method (*Pearse* 1960)

Glycogen and Glyco Lipoprotein

1
cogen
cent
se cause complete loss of glycogen—particularly in embryonic cells (*Andersen* 1963 a)

Mucopolysaccharides

verify these findings from a cytochemical point of view, as well as to map out the chemical processes which take place in these lysosomes

MATERIAL

The material comprises 6 human foetuses removed by Caesarean section in connection with a well founded interruption of pregnancy Based on crown rump lengths the material is divided up as follows 53 mm 62 mm, 119 mm, 136 mm 151 mm and 172 mm The foetuses were measured prior to fixation in a supine position unnecessary stretching having been avoided Material from the jejunum as well as from the ileum has been used

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light greenish-yellow—a shade very much like that of embryonic red blood cells in unstained preparations

Particularly in the 136 mm crown-rump length foetus the particles within the individual epithelial cell on the apical part of the villus present every conceivable gradation in size—which variation in size ranges from less than one micron to more than the size of the nucleus of the cell The smallest particles are placed mostly luminally, their size increasing as the base of the cell is approached, however, with no appreciable localization to the infranuclear part of the cell

In the early stages, glycogen is found in all epithelial cells of the intestine from the floor of the crypts to the top of the villi, but in the later stages glycogen is only seen in the cells on the top of the villus, both in supra- and infranuclear position The particles are predominantly centrally lodged in the supranuclear glycogen fraction and show maltase and salivaresistant PAS-positivity (Fig 1), but they show no reaction with Schiff's reagent without previous treatment with periodic acid

The reaction for acid phosphatase shows distinct localization of this enzyme to the cytoplasmic particles, particularly at incubation of shorter duration (Fig 2) At prolonged incubation a weaker reaction is seen luminal to the particles, and at still longer incubation reaction is also seen in the rest of the cytoplasm of the epithelial cells, both in the crypt and on the top of the villus

The localization of unspecific carboxyl-esterase is in perfect agreement with the corresponding findings in respect of acid phosphatase with distinct nodular accumulation of the final reaction product in the cytoplasmic particles (Fig 3)

The particles show marked amino peptidase activity (Fig 4), and further a weaker activity is seen in the most luminal part of the cytoplasm in the epithelial cells—especially at prolonged incubation

Alkaline phosphatase activity is seen only within a narrow zone in the extreme luminal part of the epithelium, and only in the epithelial cells on the villi, never in the crypts Concerning the villi, the reaction for alkaline phosphatase is increased with approximation to the top of the villus On the other hand, nodular accumulations of the final reaction product as a sign of the presence of the enzyme in the particles are never seen

Succinic dehydrogenase activity is seen in all epithelial cells, and is localized to their mitochondria, the reaction being most marked in the supranuclear parts of the cells

In contradistinction to the rest of the enzymes demonstrated, the lactic dehydrogenase activity is found to be uniformly distributed in the cytoplasm of all epithelial cells (Fig 5) right from the crypt to the top of the villus

Regarding the presence of proteins in the particles, it should be stated that, using the special amino acid reactions a positive reaction for SH

Bile Pigments

1. The Gmelin-reaction for bilirubin and haematoidine (*Pearse* 1960)
2. Kutlik's ferri-iron method for bilirubin (*Pearse* 1960)
3. Stein's iodine method for bile pigments (*Pearse* 1960)

The Demonstration of Iron

1. Perl's method for ferri-iron (*Pearse* 1960).
2. Turnbull-blue method for ferri- and ferro-iron (*Pearse* 1960)

Control Experiments

1 In all enzyme reactions control sections were used which had been inactivated in steam at 90–100° C for 10–15 min and, as a further control, sections were incubated in the incubation medium without substrate

2 In order to test the presence of SH-groups, mercaptide block with phenyl mercurichloride in propanol (*Lillie* 1954) was performed, as in very rare cases an unspecific binding of mercury orange to lipid substances may occur

3 As a test for the presence of tyrosine, iodination of the tyrosine side chain with Gram's iodine and ammonia (*Pearse* 1960) was carried out This procedure will block the reaction of the tyrosine with the Millon reagent

4 As a control to the ninhydrin Schiff reaction for NH_2 groups in protein (α amino acids), sections were included which had not undergone deamination with ninhydrin

5 In order to separate the glycogen reaction with periodic acid Schiff from other PAS positive substances, maltase- as well as saliva digestion of control sections was carried out prior to the performance of the PAS reaction

6 In order to liberate masked iron, if any, unmasking was performed by means of hydrogen peroxide (*Pearse* 1960)

7 As a further test of the presence of the smaller intra epithelial particles, phase contrast microscopy was carried out

8 With a view to demonstrating cytoplasmic RNA, control digestion with ribonuclease *ad modum Lillie* (1954) was carried out, a four times crystallized, pure, salt-free, and protease free ribonuclease preparation (Fluka) being used

OBSERVATIONS

Already in the foetus of 53 mm crown-rump length, small, round inclusion particles are seen in the supranuclear part of the epithelial cells of the intestine, particularly towards the top of the villi, while the amount of particles as well as their size decrease and finally completely disappear at the base of the villi At no time of development are these particles seen in the cells of the crypts

During the subsequent stages of development there is an increase in the amount and the size of the particles, which in the middle section of the small intestine in the 136 mm foetus (c -r) may gain a size which far exceeds that of the nuclei of the epithelial cells, and almost completely fill the luminal part of the cells The sections from the 172 mm foetus subjected to examination did, however, contain particles of a smaller size than the nucleus of the cell

Further, on oral anal approximation there is an increase in the size and amount of the above particles, which increase, however, is reversed in the anal part of the ileum which is seen to be greatly distended by meconium of a green colour

With increasing size the natural colour of the particles becomes more pronounced, and in unstained preparations the colour is seen to turn a

light greenish-yellow—a shade very much like that of embryonic red blood cells in unstained preparations

Particularly in the 136 mm crown rump length foetus the particles within the individual epithelial cell on the apical part of the villus present *every conceivable gradation in size*—which variation in size ranges from less than one micron to more than the size of the nucleus of the cell The smallest particles are placed mostly luminally, their size increasing as the base of the cell is approached, however, with no appreciable localization to the infranuclear part of the cell

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In contradistinction to the rest of the enzymes demonstrated, the lactic dehydrogenase activity *is found to be* uniformly distributed in the cytoplasm of all epithelial cells (Fig 5) right from the crypt to the top of the villus

Regarding the presence of proteins in the particles, it should be stated that using the special amino-acid reactions a positive reaction for SH-

groups (cysteine) with mercury orange has been found (Fig 6), since the mercaptide block shows complete loss of reaction with this reagent and thus excludes unspecific reactive binding to lipid compounds The reaction with mercury orange is seen in particles of all sizes

Similarly, all particles show positive reaction for tyrosine (Fig 7) with Millon's reagent—a reaction which is completely blocked by iodination

The Sakaguchi-reaction for arginine is positive only in particles of medium and large size, the latter showing the strongest reaction

With ninhydrin-Schiff for α-amino acids (Lysine a o) a marked histochemical distinction is achieved between the different sizes of the particles, the smaller particles being negative, whereas the medium-sized show a weak, the large particles a strong reaction (Fig 8)

Both the copper-phtalocyanine method and the Nile-blue method for phospholipids show positive reaction in the particles, particularly in the small and medium-sized ones, while the large ones show no or only slight reaction

Lillie's Nile-blue method for lipofuscins shows a very strong reaction in the large particles (Fig 9), while the staining of the smaller ones is slightly weaker On the other hand, Gomori's chromalun-haematoxylin reaction for lipofuscins shows only moderate reaction or none at all

As to acid-fastness properties, all particles show positive reaction with the strongest reaction localized to the largest particles (Fig 10)

No reaction is seen for cholin-containing lipids and the reactions for ferro- and ferri-iron are negative, even after attempted ionization of masked iron Neither is there any reaction for bile pigments in the particles mentioned

Neither Alcian blue nor toluidine blue shows signs of the presence of mucopolysaccharides in the cytoplasmic particles, which with the metachromatic reaction appear strongly green

Chromalun gallocyanin staining for ribonucleinates gives no result in the particles, whereas the epithelial cells in the crypts show a strong reaction for cytoplasmic RNA, which disappears on ribonuclease digestion The epithelial cells of the villi, particularly on the top, show slight reaction, probably as a result of the large glycogen content in these cells, since the glycogen takes up large parts of the cytoplasm This is also

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Figs 1 2

Fig 1 Transverse section of villi from the anal part of the jejunum of a 136 mm crown rump f etus The intra epithelial cytoplasmic particles of varying sizes all show a PAS positive reaction Very intense PAS positivity in the goblet cells PAS staining × 350

Fig 2 Acid phosphatase activity in the intra epithelial cytoplasmic particles The jejunum from a foetus of 136 mm crown rump length Naphthol AS BI phosphate method No counterstain × 250

1

2

in agreement with collaterally performed electron microscopic studies (*Bierring et al* 1963).

A few supplementary examinations of the basophilia of the particles show negative reaction with haematoxylin; on the other hand the particles give negative reaction with aqueous 1 per cent solutions of eosin and neutral red.

CONCLUSION AND DISCUSSION

From a histochemical point of view the results suggest that the particles in question must be regarded as lysosomes. This assumption is particularly borne out by their content of acid phosphatases, unspecific carboxyl-esterases, and amino-peptidase, since especially the two former components by many authors are held to be the most reliable indications that the diagnosis of lysosomes is correct

At the same time the results clearly show that metabolic processes take place in the lysosomes, since the smaller, luminal particles show positive reaction for phospholipo-proteins, while the larger ones show signs of the conversion of these substances in the direction of lipofuscins The great variation in sizes of particles in the luminal basal direction inside the cells probably indicates a fusion of smaller particles so as to form larger and more basally located ones—an assumption which is in perfect harmony with the above collaterally performed electron microscopic studies

The positive reaction for lipofuscins is in strong support of the lysosome assumption, since pigments which accumulate or form in a cell are first seen inside lysosomes (*Barka & Anderson* 1963) Lipofuscins are autooxidation products from phospholipids and unsaturated fats, and may contain protein components (*Pearse* 1960) The same author has drawn up a diagram for the conversion into lipofuscins and has indicated the various histochemical tests which become positive during such conversion He further found that lipofuscins in nerve tissue are always attended by activity of acid phosphatases and unspecific carboxyl-esterase

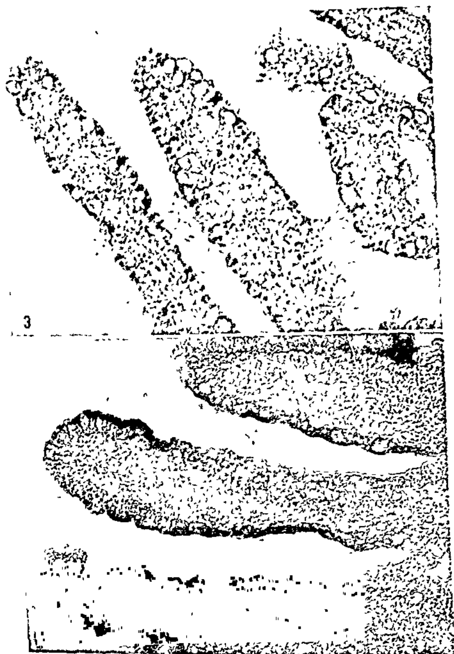
A comparison between the data obtained in the present study and Pearse's diagram shows that, as far as the larger particles are concerned, they contain components which are placed somewhere around the border between the first and the middle third in the diagram The reactions of the particles mentioned allow of such a placing, since they are

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Figs 3 4

Fig 3 Activity of unspecific carboxyl esterase in the intra epithelial cytoplasmic particles in the jejunum Foetus of 172 mm c r length Naphthol AS acetate method No counterstain × 250

Fig 4 The villi from the jejunum showing both nodular and more diffuse accumulations of the final reaction product of the L leucyl naphthyl-amide hydrochloride method for amino peptidase Crown rump length 172 mm × 250


3

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16

PAS-positive, acid fast, pigmented, and show positive reaction with Lillie's Nile-blue method for lipofuscins Schmorl's reaction, which becomes positive during the early stages of the oxidative conversion of the lipofuscins owing to the formation of reducing groups, has not been performed in the present study, since the free SH-groups demonstrated in the particles also give positive Schmorl's reaction The particles show only moderate basophilia, which can only be demonstrated by means of toluidine blue, whereby the particles assume a green colour This green staining must be regarded as a mixture of two colours, namely the natural, yellowish colour of the particles plus their orthochromatic (blue) staining with toluidine blue, since "negative" or bathochrome (green) metachromasia is otherwise known only in connection with nucleinates, *e g* in sperm heads (*Baker* 1958), and in embryonic chondrocytes (*Andersen* 1962), and probably shows a relation to *Bank & Bungenberg de Jong*'s observation (1939) that nucleinates in a certain colloidal state can turn toluidine blue into green *in vitro* In the present study nucleinates can be excluded, since the chromalun-gallocyanine staining was negative *Pearse* (1960) reports, however, that melanin is stained green by thiazine dyes as thionine, azure A and toluidine blue, which, according to *Lillie* (1955), probably is due to the content of sulphonic acid groups or ester sulphates in melanins However, melanins can be excluded in the present case as shown with Lillie's Nile blue method

Although the lipo-protein membrane of lysosomes gives positive PAS-reaction (*Barka & Anderson* 1963), this must, however, be assumed to be the cause of only a minor part of the PAS positive reaction which is seen in the present case and especially in the stages of development where the particles achieve a large size there can be no doubt that also the particle-content itself is positive

In the larger particles the negative reaction with Menschik's Nile blue method for phospholipids also suggest lipofuscins, since the subsequent acetone treatment of the Nile blue stained sections will immediately decolourize lipofuscins (*Lillie* 1956) Only at pH values above 3 is the Nile-blue staining acetone resistant, which according to Lillie indicates carboxylic acids as the reactive groups in forming the tissue dye complexes

The actual structural composition of the lysosomes, with the excep-

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Figs 5–6

Fig 5 Uniformly distributed cytoplasmic activity of lactic dehydrogenase in the intestinal epithelium The ileum from a foetus of 172 mm c r length DL lactate – Nitro BT method × 250

Fig 6 Reaction for free SH groups in the intra epithelial cytoplasmic particles from the jejunum Transverse section of the villi with very strong reaction for SH groups in the red blood cells Crown rump length 136 mm Mercury orange staining × 450


15
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7

8

tion of their enzyme content, is not known (*Barka & Anderson* 1963), but they contain different components and are the seat of metabolic processes of various kinds Thus, in some lysosomes ferritin and haemosiderins (*Essner & Novikoff* 1960) have been demonstrated, while, as already mentioned, others contain lipofuscins In the present case, the negative reactions for ferri- and ferro-iron, even after attempted ionization of masked iron, show that these intestinal epithelial lysosomes do not contain ferritin and haemosiderins

The positive reaction of the smaller particles for phospholipids as well as the positive reaction with mercury orange and Millon's reagent shows that these particles contain phospholipo-protein complexes The complete loss of the particles with ethanol containing fixatives points in the same direction, since it is well known that ethanol causes a splitting up of lipo-protein components A splitting of the lipo-protein membrane of the particles owing to the ethanol, however, can not be excluded as being the cause of the loss

The negative reaction for arginine and α amino-acids in the smaller particles and the positive reaction for these in the larger ones suggest that during the conversion of the phospholipoproteins into lipofuscins, changes in the form of unmasking of the active end-groups in the amino-acids take place

The question of the origin of the substances which are converted into lipofuscins in the lysosomes is still open, in this connection it would be natural to direct the attention towards the intestinal content, which, particularly in the ileum, is abundant, consisting in part of muco polysaccharides from the numerous goblet cells, which with PAS-staining and Alcian blue staining show distinct signs of secretion into the intestinal lumen, and in part of swallowed amniotic fluid Further, in the intestinal lumen nodular masses are seen to stain weakly β metachromatic with toluidine blue and to exhibit moderate PAS-positivity, macroscopically these masses are of a strong green colour, no doubt ascribable to bile pigments These meconium masses are usually assumed to consist of swallowed *vernix caseosa* as well as remnants of exfoliated epithelial cells from the alimentary canal and products of secretion from the same source

It is a fact that a strong absorption from the intestinal lumen into the

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Figs 7–8

Fig 7 Intra epithelial cytoplasmic particles from anal part of the jejunum show [illegible] in the goblet cells Crown rump length [illegible]

Fig 8 [illegible] ithelial cytoplasmic particles with the strongest reaction in the larger ones No reaction in the goblet cells Anal part of the jejunum from a foetus of 136 mm c r length Ninhydrin Schiff method No counterstain × 220

epithelium takes place, since the electron microscopic studies (*Bierring et al* 1963) show a great pinocytotic activity emanating from the plasma membrane between the microvilli This pinocytosis explains the previously mentioned moderate activity of acid phosphatases luminal to the lysosomic particles, since this (these) enzyme(s) appear(s) to influence pinocytotic processes (*Burstone* 1962) On the other hand, the acid phosphatase activity in the cells of the intestinal crypts must probably be ascribed to the protein synthesis here (proliferation of cells is only seen in the crypts), since this enzyme is of apparent significance for this synthesis (*Burstone* 1962)

The negative reaction of the lysosomes with Alcian blue suggests that the mucopolysaccharides (mucus) are not absorbed from the intestinal lumen—at least not in an unconverted form, since Alcian blue usually stains goblet cell granules as well as the mucus in the intestinal lumen Also the reactions for bile pigments are negative in the cytoplasmic particles, the natural colour of which can thus hardly be ascribed to pinocytosed bile pigments, but must be due to the growing pigmentation of the lipofuscins Among other things, pinocytosis of *vernix caseosa* and cellular debris, which mainly consists of phospholipo-proteins from the different membranes of the cells, remain to be taken into consideration

The alkaline phosphatase activity in the extreme luminal part of the epithelium of the villi completely resembles the corresponding phenomena in the adult intestine, in which the enzyme is localized to the microvilli (*Burstone* 1962), and probably is significant for the glucose resorption by converting glucose into glucose phosphate

It is remarkable that the inclusion bodies have not been mentioned in the common embryological text books (*Minot* 1892, *Keith* 1933, *Patten* 1953, *Starck* 1955, *Arey* 1958, and *Hamilton, Boyd* & *Mossman* 1959) This is probably related to the use of ethanol-containing fixatives which, as previously mentioned, cause a complete loss of these particles

Many problems concerning these particles still remain unsolved, they are now being investigated in a combined histochemical-electron microscopic study The investigations concerned are directed primarily towards the ultrastructure and cytochemistry of the epithelium at the

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Figs 9 10

Fig 9 Transverse section of villi from the anal part of the jejunum Very strong reaction for lipofuscins in the large intra epithelial cytoplasmic particles while the smaller ones shows a weaker reaction Crown rump length 136 mm Lillie s Nile blue method for lipofuscins × 220

Fig 10 All intra epithelial cytoplasmic particles showing acid fastness properties Anal part of the jejunum from a foetus of 136 mm crown rump length Long Ziehl Neelsen method for the acid fastness of lipofuscin × 220


10

time when the first particles appear in the epithelium, and further towards the chemical data of the particles during the different stages of development until their disappearance around the 7th foetal month

SUMMARY

In a material comprising 6 human foetuses with crown-rump lengths varying from 53 mm to 172 mm (11th 20th menstrual week) round, intra-epithelial cytoplasmic particles of varying size are seen in the jejunum and the ileum

The size and the amount are increased with approximation to the top of the villus as well as with oral-anal approximation, but decrease in the anal part of the ileum which is dilated by green meconium masses At no time are the particles seen in the epithelium of the crypts

The particles show activity for acid phosphatases, unspecific carboxyl esterase, and amino-peptidase, whereas alkaline phosphatase activity is seen only in the extreme luminal part of the epithelium

All particles show positive reaction for SH-groups and tyrosine, while the larger particles show positive arginine reaction and are positive with ninhydrin-Schiff for α-amino-acids

Similarly, the smaller particles show positive reaction for phospholipids, while the larger ones show signs of the presence of lipofuscins, these particles being strongly positive with Lillie's Nile blue method for lipofuscins and exhibiting acid-fastness properties

All particles are maltase-resistent PAS positive with the strongest reaction in the larger particles, and all particles are stained strongly green by toluidine blue In unstained preparations the larger particles have a yellowish green colour, which is more intense in the largest particles

With ethanol-containing fixatives there was a complete loss of the cytoplasmic particles

In the particles no positive reaction is seen for bile pigments or iron

Based on the results obtained, the conclusion is drawn that the particles in question most likely are lysosomes, in which absorbed phospholipo-proteins from the meconium are converted into lipofuscins, and that the larger particles apparently are formed by a fusion of smaller ones, which is in good agreement with collaterally performed electron microscopic studies

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BILATERAL RENAL CORTICAL NECROSIS AND THE GENERALIZED SHWARTZMAN REACTION

I Review of Literature and Report of Seven Cases

By

FREDRIK SKJORTEN

Received 20 xii 63

The first descriptions of bilateral renal cortical necrosis were given by *Friedlander* (1883) and *Juhel-Renoy* (1886), but until 20 years ago it was a rarely diagnosed entity *Duff & More* (1941) were able to collect 79 cases from the literature and from personal material In their study the association with pregnancy was of dominating importance

In the more recent literature many reports of bilateral renal cortical necrosis have appeared It does occur in both sexes and all age groups A number of cases have been seen in children (*Eskeland & Skogrand* 1959, *Bohle & Krecke* 1959) Attention has been centered on the similarity between bilateral renal cortical necrosis and the generalized Shwartzman reaction.

In the present paper the pertinent literature regarding the pathogenesis of bilateral renal cortical necrosis is reviewed and seven personal cases are reported Some previously unnoticed features of the intravascular fibrin precipitates in bilateral renal cortical necrosis are pointed out These features will be discussed further in a subsequent paper

AETIOLOGY AND PATHOGENESIS

All authors who discuss this problem agree that a vascular occlusion in the renal cortex is the direct cause of bilateral renal cortical necrosis In more recent reports there seems to be two separate schools of thought regarding the pathogenesis of this vascular occlusion

One school of thought is represented by *Trueta & al* (1947) In a monography on the renal circulation they reported extensive experiments in rabbits showing that a vascular spasm in the juxtamedullary layer of the renal cortex could shunt the blood away from the renal cortex and thus cause ischaemia This vascular spasm could be induced by nervous stimulation, by drugs, and by the injection of staphylococ-

cus toxin The conclusion was that renal cortical necrosis is caused by renal ischaemia due to vascular spasm and that thrombosis in cortical vessels is secondary to vasospasm

Sheehan & Moore (1952) made a very detailed study of 34 cases of bilateral renal cortical necrosis occurring in pregnant women with premature separation of the placenta They attempted to reconstruct the chain of events leading to renal cortical necrosis on the basis of lesions seen in patients dying at different intervals after the onset of symptoms They conclude that renal cortical necrosis can only be explained by the occurrence of a vascular spasm in the renal cortex of several hours duration followed by recirculation and subsequent thrombosis of already necrotic vessels

The theory of vasospasm is also supported by the works of *Erspamer & Ottolenghi* (1953) They have shown that in rats injections of serotonin in unphysiological doses will produce bilateral renal cortical necrosis The basis for this they claim is an arteriolar spasm in the renal cortex

The second school of thought is represented by *Apitz* (1934) *McKay & al* (1953 1959) and *Bohle & Krecke* (1959) These authors claim that fibrin deposition in afferent arterioles and glomerular capillaries is the primary lesion and the cause of the vascular occlusion in bilateral renal cortical necrosis and point out the similarity to the generalized Shwartzman reaction as seen in the rabbit

McKay & al (1953 1959) point out that in cases of bilateral renal cortical necrosis one may frequently find fibrin thrombi not only in the renal cortex but also in the liver spleen pituitary and adrenal glands Less frequently thrombi are found in lungs myocardium brain and intestinal walls These findings are similar to those seen in the generalized Shwartzman reaction They are not compatible with the vasostatic theory of *Trueta & al* (1947) according to which the kidneys are the target organs

The generalized Shwartzman reaction has been extensively studied in the rabbit It is induced by two intravenous injections of bacterial endotoxin given at 24 hours interval The animals become seriously ill and frequently die 24 to 48 hours after the last injection At autopsy the characteristic finding is bilateral renal cortical necrosis Microscopically there is extensive deposition of fibrin in glomerular capillaries renal cortical arterioles and in small vessels in the liver spleen myocardium and brain (*Apitz* 1934 *Thomas & Good* 1952) A similar reaction can be produced in other species but in each the reaction has a different picture (*Gronwall & Brunson* 1956 *Hardaway* 1962) In the dog injections of endotoxin will give haemorrhagic reactions A similar reaction is seen in the rabbit when endotoxin is combined with fibrinolysin

McKay & Shapiro (1958) showed that during the generalized Shwartzman reaction there is extreme depletion of the circulating

fibrinogen, and at the same time fibrin, or a fibrin-like material, appears in the microcirculation of many organs, but most abundant in the kidneys The reaction is prevented by heparinization (*Good & Thomas* 1953) Thus it is evident that the generalized Shwartzman reaction is a cathastrophe in which intravascular coagulation plays an important part

Shainoff & Page (1960) were able to show that during endotoxin shock in the rabbit there is a six-fold increase in the serum concentration of cryo-profibrin, indicating a transformation of fibrinogen to fibrin of a similar magnitude Further studies on the action of endotoxin have been made by *Weissman & Thomas* (1962) and by *Janoff & al* (1962) They have shown that after endotoxin injections in the rabbit there is augmented release from the lysosome fraction of liver cell preparations of acid hydralases including cathepsins which are potent proteolytic enzymes There is also an increased cathepsin-concentration in the serum of endotoxin treated rabbits Information about the action of cathepsin on the fibrinogen molecule is so far lacking

Increased serum concentration of trypsin may give rise to a generalized Shwartzman reaction *Richet & al* (1960) reported nine cases of bilateral renal cortical necrosis in patients with acute pancreatitis in whom shock did not develop until after the renal complication had occurred Injections of thrombin may cause a Shwartzman reaction in the rabbit (*Lee* 1962)

Hardaway (1962) has shown that intravascular clotting, presumably caused by increased absorption of endotoxin from the intestine, may be an important factor in the pathogenesis of irreversible shock in dogs He claims that this may also be the case in humans

Why then does not a Shwartzman reaction occur more often outside the laboratory? *Lee* (1962) may have shed some light upon this point He has shown that after prior blockage of the reticuloendothelial system, one injection of endotoxin is sufficient to precipitate the generalized Shwartzman reaction, whereas normally two injections at 24 hours interval are necessary The fibrin produced by the first injection will be phagocytized, and a massive systemic fibrin precipitation can only occur after saturation of the reticuloendothelial system This may explain why the 24 hour interval between injections is essential for the production of the generalized Shwartzman reaction

There has been some disagreement as to the nature of the material which is deposited intravascularly in the generalized Shwartzman reaction *Pappas & al* (1958) studied the material in the electron microscope and found that it is a fibrin-like material but not true fibrin *McKay & al* (1959) after immunochemical studies, and *Bohle & al* (1959) on the basis of electron microscopy, claim on the other hand that it is true fibrin

Thus, most authors believe that bilateral renal cortical necrosis is caused by Shwartzman reaction The generalized Shwartzman reac-

tion may in the experimental animal be produced by injections of endotoxin or by injections of proteolytic enzymes In both cases there is increased transformation of fibrinogen to fibrin or a fibrin-like material which is deposited in the microcirculation of many organs, but most evident in the kidneys where it may cause vascular occlusion and cortical necrosis The reaction is facilitated by blockage of the reticulo-endothelial system and prevented by heparin

PERSONAL CASES

At Ullevål Hospital, Institute of Pathology, we have seen seven cases of bilateral renal cortical necrosis in the three-year-period July 1. 1959 —June 30 1962 The total number of autopsies performed in this period was 5771

Autopsies were performed 12 to 36 hours post mortem. Tissue blocks were fixed in 4 per cent formalin and embedded in paraffin Sections were stained with haematoxylin and eosin, phosphotungstic acid haematoxylin (PTAH) and with Lendrum's acid picro-Mallory method for demonstration of fibrin (*Lendrum* 1949)

Case 1 H K.B ♀, 45

Chronic pulmonary tuberculosis, alcoholism, and drug addiction Last admission one week before death with a complaint of dyspnoea and increasing signs of cardio pulmonary insufficiency Urine analysis was normal on admission Three days before death she became more dyspnoeic and cyanotic, and appeared critically ill On the day of death she was comatose and had convulsions Laboratory tests K 8 m eq/l CO_2 13 m eq/l BUN not taken, urinary output not recorded Autopsy Chronic tuberculosis in left lung Both kidneys enlarged, weight 390 g The surface was smooth with patchy grey areas and small haemorrhages in the cortex, the medulla appeared normal *Other organs showed no gross changes Microscopic* examination of the kidneys showed bilateral cortical necrosis with a narrow intact [illegible] thrombi [illegible] ser fibrin [illegible] osition in [illegible] ny small, [illegible] ibrin

Case 2 H A ♂, 45

Previously healthy After heavy meals nausea, vomiting, fever and numbness in legs and fingers and increasing weakness After five days anuric, two days later admitted to medical department BP 170/85 BUN 285 mg/100 ml, WBC 17300, CO_2 15 [illegible] Cardiac hypertrophy, weight [illegible] nal cortex showed confluent [illegible] ther organs showed no gross [illegible] l bilateral cortical necrosis [illegible] om spleen and liver

Case 3 H N ♂ 66

[illegible] BUN 103 mg/100 ml A urine sample contained blood and protein He expired on the day of admission Autopsy Heart weight 510 g Marked coronary sclerosis with stenosis but no thrombi in major arteries. The myocardium showed a large



Figs 1–2

Fig 1 Case 1 Extensive fibrin deposits in glomerular capillaries afferent arterioles and interlobular arteries Necrosis and granulocyte-infiltration PTAH, 63 ×

Fig 2 Case 5 Hyaline microthrombi in pituitary capillaries Lendrum's stain 1000 ×

area of recent infarction The kidneys weighed 330 g The cut surface showed multiple white patches in the cortex The medulla appeared normal Microscopic examination of the heart showed a recent infarct In adjacent areas thrombi were found in capillaries and small venules The kidneys revealed bilateral cortical necrosis similar to case 1 No other sections were available

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liver and heart

Case 5 L.W ♀ 41

F ch ldren Symptoms of toxaemia during pregnancies three and four years

There was vaginal bleeding and signs of premature separation of the placenta The same day she delivered a stillborn baby weight 1500 g There was considerable haemorrhage during delivery Subsequently she became anuric and was transferred to medical department Hb 7 2 g/100 ml WBC 29900 BUN 117/100 ml BUN rose *rapidly and the urinary output remained at 15 20 ml/24 hours Four haemodialyses* were performed during the next four weeks She died from septicaemia Blood cultures grew staphylococcus aureus Autopsy Small abscess in left lung 900 ml of purulent pericardial effusion The heart weighed 520 g and showed left ventricular hypertrophy The right kidney was small and shrunken weight 50 g The surface was granular and the cut surface showed several areas of cortical necrosis The medulla was unaffected and the renal vessels were patent on this side The left kidney weighed 5 0 g and was of a dark brown colour The cut surface showed complete necrosis of the cortex A narrow subcapsular zone of renal tissue appeared intact The medulla was pale and swollen with necrosis in one area There were thrombi in one branch of the renal vein and all branches of the renal artery The

found in pituitary intracerebral and dural vessels (Fig 2) Thrombi were found in sections from the heart liver lungs and adrenal glands

Case 6 A L R ♀ 22

Previously healthy except for an attack of ureteral colic One week prior to death acute abdominal pain and urinary frequency admitted to surgical department with a complaint of right flank pain A urine sample contained proteine many WBC and



Figs 3–4

Fig 3 Case 6 Glomerulus showing fibrin deposits in vas afferens and several capillaries Two dense areas resembling hyaline microthrombi (arrows) PTAH, 160 ×

Fig 4 Case 6 Hyaline microthrombus fairly large distending small intracerebral venule Note characteristic vacuoles in center of thrombus Lendrum s stain 400 ×

... but expired next day Autopsy Considerable cerebral oedema The kid...

Case 7 S O ♂, 54 sailor
Had been sent home from overseas because of alcoholism Had been drinking ... three ... Three days prior to death on arrival in port he

methanoltest negative On admission he was ...
he was anuric, and expired two days after admission Autopsy ...
haemorrhages in skin and internal organs marked pulmonary oedema Fatty metamorphosis of liver The kidneys weighed 510 g The surface was smooth of a red

not as abundant as in the other cases No thrombi were found in sections from lungs liver spleen brain and heart

DISCUSSION

These seven cases present no uniform clinical picture Obviously, the chain of events leading to the terminal disaster is different in all cases and impossible to reconstruct retrospectively

The main features of the gross and microscopic pathology of bilateral renal cortical necrosis have been well documented by many authors (*Juhel Renoy* 1886, *Duff & More* 1941, *McKay & al* 1953) and need not be discussed in detail here

Among the seven cases presented in this paper, there were four females and three males Only one of the female patients was pregnant in contrast to previous reports in which a majority of the patients were pregnant (*Duff & More* 1941, *Sheehan & Moore* 1952, *McKay & al* 1953) Cases 1 3 4 and 6 had extensive fibrin precipitations in glomerular capillaries, afferent arterioles and interlobular arteries of the renal cortex (Fig 1) They did also, however, show intravascular fibrin precipitations in the pituitary, adrenal glands, heart and brain (Fig 2 and 4) As already pointed out by *McKay & al* (1953) this finding is compatible with the concept of a generalized Shwartzman reaction as the pathogenetic mechanism of bilateral renal cortical necrosis It can not be explained by the vasospastic theory of *Trueta & al* (1947)

Case 2 had renal findings identical with the cases mentioned above, but sections were only available from kidneys, spleen and liver, and for this reason no definite conclusion can be made regarding the pathogenesis in this case

Case 5 is somewhat complicated in that this patient had toxaemia of pregnancy and developed anuria subsequent to premature separation of the placenta Four weeks later she developed septicaemia The possibility cannot be excluded that a vasospasm mediated from the uterus (*Saurio* 1957) caused the renal ischaemia leading to renal cortical necrosis in this case However, a generalized Shwartzman reaction is equally possible We consider the thrombosis of the left renal artery and vein secondary to cortical necrosis since no thrombi were seen in the right renal artery and vein and the right kidney also showed cortical necrosis

In case 7 there was extensive cortical necrosis, but few thrombi were found in glomeruli and cortical vessels In other organs, including the brain, none were found This patient had been subject to trichlorethylene poisoning A direct toxic effect on the renal parenchyma with secondary thrombosis can not be excluded in this case

Previous reports (*McKay & al* 1953, *Bohle & Krecke* 1959) describe the glomerular fibrin precipitates in bilateral renal cortical necrosis as dense, homogenous In cases 1 6 we have found that the fibrin precipitates consists of a dense center surrounded by a looser mesh of fibrin threads with a morphology similar to thrombi found in other small vessels The center is of a more hyaline appearance, it is round or oval and stands out from the surrounding fibrin mesh (Fig 3)

In pituitary capillaries as well as in dural and cerebral vessels, small, dense, eosinophilic structures were found, very similar in appearance to the dense center of the glomerular fibrin precipitates They had the staining characteristics of fibrin (Fig 2 and 4) We therefore propose to call them hyaline microthrombi To our knowledge, hyaline microthrombi have not been described previously

The morphology and distribution of the fibrin precipitates in bilateral renal cortical necrosis and their relationship to the mechanism of the generalized Shwartzman reaction will be discussed in a subsequent paper

SUMMARY

The recent literature on bilateral renal cortical necrosis is reviewed with special emphasis on papers dealing with the pathogenesis of this entity It is concluded that most authors seem to favour the generalized Shwartzman reaction as the pathogenic mechanism in bilateral renal cortical necrosis

Seven fatal cases of bilateral renal cortical necrosis are reported In four, possibly five, of these cases the renal lesions may have been precipitated by a generalized Shwartzman reaction One cases may have a direct toxic aetiology and in one case no conclusion can be made regarding the pathogenesis

The fine morphology of glomerular thrombi in bilateral renal cortical

necrosis is briefly discussed The similarity of the center of these thrombi to hyaline microthrombi found in pituitary and other vessels is pointed out

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The fine morphology of glomerular thrombi in bilateral renal cortical

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Head Professor Kristen Arnesen, M D

BILATERAL RENAL CORTICAL NECROSIS AND THE GENERALIZED SHWARTZMAN REACTION

2 *Observations on the Morphology of Fibrin Precipitates and Discussion of the Mechanism of Thrombus Formation*

By

FREDRIK SKJORTEN

Received 20.xii 63

In a previous paper seven cases of bilateral renal cortical necrosis were reported (*Skjorten* 1964) The pathological findings in these cases were discussed in relation to the pathogenesis of the renal lesions It was concluded that in four, possibly five, cases the renal lesions might have been precipitated by a generalized Shwartzman reaction, in accordance with most current papers dealing with this subject

The generalized Shwartzman reaction is essentially a severe clotting disturbance which leads to massive intravascular coagulation, most evident in the kidneys Fibrin is precipitated in glomerular capillaries, afferent arterioles and interlobular arteries of the renal cortex This leads to ischaemia of renal cortical tissue The literature dealing with the clotting disturbance present in the generalized Shwartzman reaction has been reviewed in a previous paper (*Skjorten* 1964) In the present paper the morphology and distribution of fibrin precipitates in cases of bilateral renal cortical necrosis will be discussed further

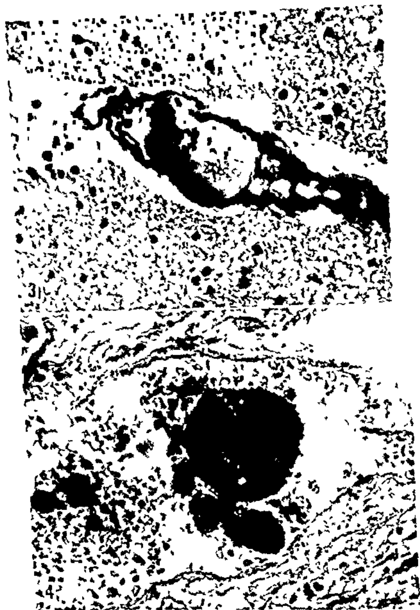
MATERIAL AND METHODS

The observations reported below are based on findings in cases of bilateral renal cortical necrosis reported in a previous paper (*Skjorten* 1964) Autopsies were performed 12 to 36 hours post mortem Tissue blocks were fixed in 4 per cent formalin and embedded in paraffin Sections were stained with haematoxylin and eosin, phosphotungstic acid haematoxylin (PTAH) and Lendrum's acid picro Mallory method for demonstration of fibrin (*Lendrum* 1949)

OBSERVATIONS

Detailed study of the glomerular fibrin precipitates reveals that they have a quite characteristic appearance Most authors describe them as dense, homogenous (*McKay & al* 1953, *Thomas* 1959) This, we have

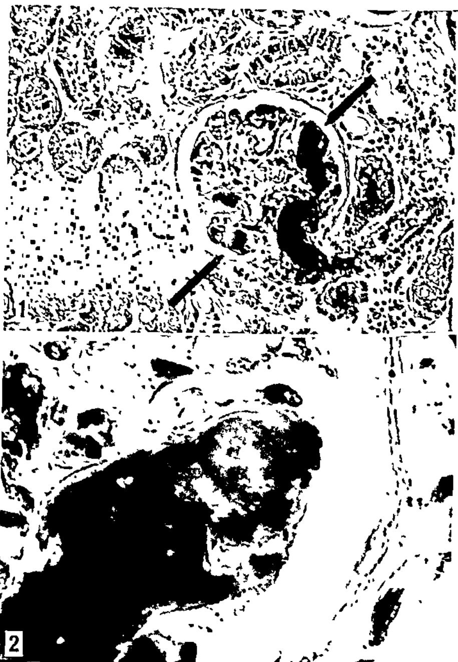
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Figs 3–4

Fig 3 Hyaline microthrombus, *fairly large, distending* small intracerebral venule Note characteristic vacuoles in center of thrombus, compare Fig 2 Lendrum's stain 400 ×

Fig 4 Small vein containing *several microthrombi* which appear less compact and have a slightly irregular outline PTAH, 400 ×



Figs 1–2

Fig 1 Glomerulus showing fibrin deposition in vas afferens and several capillaries. Two dense areas resembling hyaline microthrombi (arrows) PTAH 160 ×

Fig 2 Detail from Fig 1 showing glomerular capillary containing dense area resembling hyaline microthrombus Note sharp outline and small central vacuoles PTAH 1000 ×



Figs 7 8

Fig 7 Case 1 Limited fibrin deposition in five out of six visible glomeruli Note patent vas afferens No necrosis PTAH, 63 ×

Fig 8 Case 2 Detail of glomerulus with small fibrin deposits in two capillaries (arrows) Lendrum's stain, 1000 ×





Figs 5 6

Fig 5 Coronary vein showing hyaline microthrombi (arrows) within loose clot PTAH 160 ×

Fig 6 Hyaline microthrombi partly surrounded by platelets Lendrum's stain 1000 ×



Figs 7 8

Fig 7 Case 1 Limited fibrin deposition in *five out of six* visible glomeruli Note patent vas afferens No necrosis PTAH 63 ×

Fig 8 Case 2 Detail of glomerulus with small fibrin deposits in two capillaries (arrows) Lendrum's stain, 1000 ×

found, is an incomplete description In many glomerular capillaries we find that the fibrin precipitates consist of a dense, oval or round center which is surrounded by, and often seem to fuse with, a mesh of tightly packed threads which may extend into and occlude afferent arterioles and interlobular arteries in the renal cortex In some glomeruli we find precipitates in which the dense center has a distinct outer limiting surface, giving the impression of a round corpuscle and standing out from the surrounding material In the dense center one may find tiny vacuoles

The dense center of the precipitates varies somewhat in its staining characteristics In the PTAH stain it may stain like ordinary fibrin, or it may have a lighter, more brownish colour and thus resemble fibrinoid In Lendrum's acid picro-Mallory stain it always stains red The surrounding threadlike mesh always stains like ordinary fibrin, and it is similar in appearance to thrombi found in other small vessels At times it may contain platelets and leucocytes (Fig 1 and 2)

In cases of bilateral renal cortical necrosis careful search may reveal hyaline, eosinophilic, round bodies in the microcirculation of various organs These bodies have a diameter of a few to 20–30 μ and are similar in appearance to the hyaline center of the glomerular fibrin precipitates, containing similar tiny vacuoles and having similar staining characteristics At times these round bodies take the PTAH stain like regular fibrin, at times they have a lighter, more brownish colour, similar to fibrinoid In Lendrum's acid picro-Mallory stain they are always red (Fig 3)

Occasionally one may find round bodies which are less dense Individual fibrin threads may be differentiated within their mass (Fig 4) These bodies may possibly be about to form Sometimes the round bodies may be seen in the center of loose clots (Fig 5), at other times one may find heaps of platelets adhering to the surface of round bodies (Fig 6)

In order to be able to detect the round bodies it is necessary to search sections systematically with high magnification Even though the round bodies may be seen in haematoxylin and eosin stained sections, Lendrum's stain is preferred because it gives better contrast to other tissue components

DISCUSSION

How and why do these round bodies form, and what is their relation to bilateral renal cortical necrosis? *Alkjaersig & al* (1962) state that in abnormal plasma proteolytic states defective clots will form because of defective polymerization of fibrin *Bang & al* (1962) studied these defective clots by electron microscopy They found inhibition of fiber formation and appearance of undifferentiated fibrin aggregates which at times, judging from their micrographs, had an oval or round appearance, somewhat similar to the round bodies discussed above, but in a

smaller dimension. These bodies were incorporated into pathological clots. It is possible that the round bodies which we have observed may be condensations of such undifferentiated fibrin aggregates. At the present time however, we have no proof of this.

Hardaway (1962) has shown that in dogs, intravascular coagulation can be precipitated by endotoxin shock, incompatible blood transfusion, and haemorrhagic shock. *Weissman & Thomas* (1962) and *Janoff & al* (1962) found augmented release of intracellular proteolytic enzymes in rabbits subjected to endotoxin shock. Previously, *Shainoff & Page* (1960) had been able to show that in rabbits there is an increased tranformation of fibrinogen to fibrin during endotoxin shock.

If similar clotting disorders occur in humans during irreversible shock, one would expect to find signs of intravascular coagulation in this condition. *Hardaway & Weiss* (1962) have published two cases of intravascular coagulation after prolonged shock. We have found round bodies with the staining characteristics of fibrin in large numbers in the microcirculation of patients with bilateral renal cortical necrosis. We have also, however, found these structures in cases of septic and circulatory shock, and sometimes in cases where the underlying disease has been obscure (*Skjørten,* in preparation). We believe that the round bodies which we have observed are pathological clots, formed *in vivo,* and we propose to call them hyaline microthrombi.

A careful search of the literature has failed to reveal other reports of structures similar to the hyaline microthrombi observed by us. *Hardaway & Weiss* (1962) stated that many cases of intravascular coagulation may pass unnoticed because thrombi are small and transient. They did not comment on the morphology of these transient thrombi, and their report does not indicate that they have observed structures similar to our hyaline microthrombi.

Apparently the clotting disorder which causes the hyaline microthrombi is not always equally pronounced. At times it may cause fibrin precipitation severe enough to kill the patient under the dramatic clinical picture of bilateral renal cortical necrosis, while at other times the fibrin precipitation is so slight that it passes unnoticed. Two cases are reported which may illustrate this point.

Case 1 H O 9 60

was performed three days after admission but the patient expired next day. Additional laboratory tests: Hb 10.6 g/100 ml, WBC 8900, platelets 200000, whole

oedema. The kidneys

wed no gross signs of

Microscopic examina

vever there was wide spread fibrin precipitation intravascularly in multiple glomeruli filling several capillaries in each affected glomerular tuft (Fig. 7). No thrombi were found either

found, is an incomplete description In many glomerular capillaries we find that the fibrin precipitates consist of a dense, oval or round center which is surrounded by, and often seem to fuse with, a mesh of tightly packed threads which may extend into and occlude afferent arterioles and interlobular arteries in the renal cortex In some glomeruli we find precipitates in which the dense center has a distinct outer limiting surface, giving the impression of a round corpuscle and standing out from the surrounding material In the dense center one may find tiny vacuoles

The dense center of the precipitates varies somewhat in its staining characteristics In the PTAH stain it may stain like ordinary fibrin, or it may have a lighter, more brownish colour and thus resemble fibrinoid In Lendrum's acid picro-Mallory stain it always stains red The surrounding threadlike mesh always stains like ordinary fibrin, and it is similar in appearance to thrombi found in other small vessels At times it may contain platelets and leucocytes (Fig 1 and 2).

In cases of bilateral renal cortical necrosis careful search may reveal hyaline, eosinophilic, round bodies in the microcirculation of various organs These bodies have a diameter of a few to 20 30 μ and are similar in appearance to the hyaline center of the glomerular fibrin precipitates, containing similar tiny vacuoles and having similar staining characteristics At times these round bodies take the PTAH stain like regular fibrin, at times they have a lighter, more brownish colour, similar to fibrinoid In Lendrum's acid picro-Mallory stain they are always red (Fig 3)

Occasionally one may find round bodies which are less dense Individual fibrin threads may be differentiated within their mass (Fig 4) These bodies may possibly be about to form Sometimes the round bodies may be seen in the center of loose clots (Fig 5), at other times one may find heaps of platelets adhering to the surface of round bodies (Fig 6)

In order to be able to detect the round bodies it is necessary to search sections systematically with high magnification Even though the round bodies may be seen in haematoxylin and eosin stained sections, Lendrum's stain is preferred because it gives better contrast to other tissue components

DISCUSSION

How and why do these round bodies form, and what is their relation to bilateral renal cortical necrosis? *Alkjaersig & al* (1962) state that in abnormal plasma proteolytic states defective clots will form because of defective polymerization of fibrin *Bang & al* (1962) studied these defective clots by electron microscopy They found inhibition of fiber formation and appearance of undifferentiated fibrin aggregates which at times, judging from their micrographs, had an oval or round appearance, somewhat similar to the round bodies discussed above, but in a

vascular coagulation Apparently the hyaline microthrombi may float along in the microcirculation of many organs when intravascular coagulation takes place In the kidneys the presence of hyaline microthrombi apparently have the most serious consequences Glomerular capillaries are particularly long and tortuous Histological findings reported in this paper indicate that hyaline microthrombi may be "embolized" in glomerular capillaries When this occurs, secondary thrombosis may take place around the hyaline microthrombi, giving rise to the glomerular changes reported above The secondary thrombosis may extend into afferent arterioles and interlobular arteries in the renal cortex and give rise to bilateral renal cortical necrosis which is the "hallmark" of the generalized Shwartzman reaction (Thomas 1959) When intravascular coagulation is less severe, fewer hyaline microthrombi will be formed, only occasional glomeruli may be affected (case 2), or none at all, and the hyaline microthrombi may be an incidental finding at autopsy

SUMMARY

✓Observations on the morphology of glomerular fibrin precipitations in cases of bilateral renal cortical necrosis are reported It is pointed out that in glomerular capillaries the fibrin precipitates often consist of a hyaline center surrounded by a loose mesh of fibrin threads

In pituitary and cerebral vessels small round bodies with the staining characteristics of fibrin have been found They have an appearance similar to the hyaline center of the glomerular fibrin precipitates They

occurrence of intravascular coagula

discussed and the relationship to the generalized Shwartzman reaction is pointed out It is proposed that the hyaline microthrombus may be the primordial body in cases of intravascular coagulation

Hyaline microthrombi may be formed in varying numbers depending on the severity of the underlying clotting disturbance The possible relationship between the formation of hyaline microthrombi and the occurrence of bilateral renal cortical necrosis is discussed

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in interlobular arteries or in afferent arterioles The precipitated fibrin had a dense homogenous appearance similar to the fibrin precipitates seen in cases of bilateral renal cortical necrosis Similar fibrin precipitates were also found in intracerebral and pulmonary vessels No thrombi were found in other organs

Case 2 H M A ♀, 82

Last year increasing signs of cardiac failure, lately confined to bed Admitted to medical department in poor condition BP 155/95 Tp 36 4° C There were signs of pericarditis A urine sample contained protein Hb 8 7 g/100 ml WBC 17500, serum creatinin 1 75 mg/100 ml ECG showed auricular fibrillation She expired two days after admission Autopsy There was a small pulmonary embolus The heart weighed 510 g There was considerable coronary sclerosis, but no thrombus The interventricular septum showed a recent infarct with mural thrombi in the left ventricle The kidneys weighed 310 g and had a slightly granular surface, but no gross signs of necrosis Other organs were not remarkable to the gross view Microscopic examination The heart showed a recent myocardial infarct The liver showed signs of severe congestion with centrilobular necroses Groups of fibrin threads were found in occasional sinusoids Sections from one kidney showed a papillary carcinoma Section
some c
above,

The histological findings in case 1 at first sight appeared similar to the microscopical picture of bilateral renal cortical necrosis Closer examination revealed that there were no signs of necrosis of renal tissue Furthermore, the afferent arterioles were not subject to fibrin precipitation Many glomerular capillaries, however, contained fibrin precipitates with a dense hyaline center surrounded by a looser mesh of fibrin threads, similar to the fibrin precipitates observed by us in cases of bilateral renal cortical necrosis Fibrin precipitates were also found in the brain and the lungs In the brain they had the appearance of hyaline microthrombi

Case 2 had only a few fibrin precipitates in occasional glomeruli of one kidney These precipitates had a microscopic appearance similar to the renal fibrin of case 1 One glomerulus contained a small round body which we judged to be a hyaline microthrombus

We believe that the two cases reported above and the previously reported cases of bilateral renal cortical necrosis represent manifestations of the same clotting disturbance, but of different severity In the previously reported cases of bilateral renal cortical necrosis the clotting disturbance was very severe and caused extensive intravascular coagulation leading to a generalized Shwartzman reaction In case 1 the clotting disturbance was also quite serious and caused fibrin precipitation in the kidneys, lungs and brain However, in the kidneys the fibrin precipitation was not so extensive that it led to occlusion of the afferent arterioles in the renal cortex and thus renal cortical necrosis In case 2 the clotting disturbance was slight, limited amounts of fibrin were precipitated and the patient experienced no symptoms referable to this disorder

The reported findings have led us to believe that the hyaline microthrombus observed by us is the primordial body in many cases of intra-

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The University of Bergen, School of Medicine, The Gade Institute, Department of Microbiology, Bergen, Norway

SEROLOGICAL TYPING OF STAPHYLOCOCCUS AUREUS

6 Antibodies of Immune Sera to Strains Cowan I, Cowan II, Cowan III, Wood 46, 670, 830, 1015, 5687, and 6376

By

Received 28 ix 63

GUNNAR HAUKENES

By means of slide agglutination with absorbed and unabsorbed immune sera *Cowan* (1939) described 3 serological types of pyogenic staphylococci which comprised about 70 per cent of all strains examined Later the number of types has been extended by several investigators (1, 10, 19) Their methods differ, however, in several respects from that used by us so that a direct comparison of the results is not possible

The agglutinogens of the 3 Cowan strains and the other type strains have been studied further by *Oeding* (1953) *Stern & Elek* (1957) examined the antigenic structure of the Cowan strains by cross-absorption experiments They demonstrated at least 8 distinct agglutinogens, some

position of immune sera to strain Wood 46 and the new type strains The designation group antigen has repeatedly been used in the present article denoting antigens which occur in most or all pyogenic staphylococci There is no sharp distinction between type and group antigens, but since the latter have little or no interest for serological typing, they have not been examined further in this study Two group antigens have been described in detail, i e antigen A (*Jensen* 1958) and polysaccharide A (*Haukenes* 1962b) Both agar precipitation and agglutinin absorption experiments have shown that there are several other group antigens

MATERIALS AND METHODS

The original Cowan types I II and III were supplied by Dr *S T Cowan* London and are as follows Strain S 11 (NCTC 8530) which is type I strain S 80 (NCTC 8531)—type II and strain S 33 R 4 (NCTC 8532)—type III

Dr *Christie* s type II strain (NCTC 6128) has been supplied by Professor *J Pillet* Paris

The other type strains have been presented before

The immunization absorption and agglutination techniques are the same here as in earlier investigations (5 9, 16)

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The agglutinogens of the 3 Cowan strains and the other type strains have been studied further by *Oeding* (1953) *Stern & Elek* (1957) examined the antigenic structure of the Cowan strains by cross absorption experiments They demonstrated at least 8 distinct agglutinogens, some of which were type specific and some shared by two or all three strains

The present article deals with cross absorption experiments with the Cowan strains and immune sera and examinations of the antibody composition of immune sera to strain Wood 46 and the new type strains The designation group antigen has repeatedly been used in the present article denoting antigens which occur in most or all pyogenic staphylococci There is no sharp distinction between type and group antigens, but since the latter have little or no interest for serological typing, they have not been examined further in this study Two group antigens have been described in detail, i e antigen A (*Jensen* 1958) and polysaccharide A (*Haukenes* 1962b) Both agar precipitation and agglutinin absorption experiments have shown that there are several other group antigens

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The other type strains have been presented before

The immunization absorption and agglutination techniques are the same here as in earlier investigations (5 9 16)

TABLL 1

Agglutinability of the Type Strains in Serum Cowan I Absorbed with Various Type Strains

Strain	Serum Cowan I absorbed with the standard dose of Cowan II bacteria						
	Not re absorbed	Re absorbed with thrice the standard dose of strains					
		Cowan II	1503	28	Wood 46	1015	Cowan I
1503	+++(250)	—	—	—	+	++	—
2253	+++(250)	—	—	—?	+	—	—
28	(+)	—	—	—	—	—	—
365	+++(100)	+(10)	—	+++	+++	++	—
3647	++(50)	—	—	—	+	+	—
F 21	+(50)	—	—	—	—	+	—
17 A	+++(500)	—	—	—	—	—	—
Wood 46	(+)	—	—	—	—	—	—
Cowan I	+++(500)	+++(250)	+	++	++	++	—
Cowan II	(+)	—	—	—	—	—	—
Cowan III	+++(25)	—	—	+	+++	++	—
670	++(50)	—	+	+	++	++	++
830	+(10)	—	—	(+)	++	—	—
5687	++(100)	—	+	+	++	++	+
6376	+(25)	—	—	—	++	—	—
3189 2035 and 1015	—						

Code (+) + ++ and +++ Strength of agglutination
— No agglutination
Figures in brackets Reciprocals of agglutination titres

EXPERIMENTAL AND RESULTS

Serum Cowan I

Theoretically the following antibodies might be found in a Cowan I serum a^{γ}, m, h^{γ}, and h_2 (6, 7, 8) in addition to the new antibodies described in this article No a, m, h_2 or h_γ antibodies could be demonstrated in the immune serum used in the present investigations

Serum Cowan I was first absorbed with strain Cowan II, and portions of this serum were thereafter absorbed with each of our 18 type strains (Table 1)

I.

ganisms After re absorption with thrice the standard dose of Cowan II bacteria only strains Cowan I and 365 agglutinated Re absorption with strains 28, Wood 46 and 1015 left agglutinins to a number of type strains as shown in Table 1 It has repeatedly been observed that strains 28 Wood 46, Cowan II, and 1015 fail to absorb agglutinins which are readily removed by most other strains In the present experiment agglutinins to strains other than Cowan I, 670, and 5687 were removed by all type strains except those mentioned above, and accordingly they represent most probably group agglutinins Strains 28, Wood 46, Cowan II and 1015 also agglutinated weakly or not at all after the first absorption with strain Cowan II (Table 1)

Aside from residual group antibodies the agglutination patterns of the re absorbed sera (Table 1) indicate the presence of two distinct antibodies, one of which could easily be identified as the h_2 antibody (cf (7)), since it agglutinated strains 670 and 5687, but was blocked in live nutrient agar cultures of the other h_2 strains These strains agglutinated when mannitol salt agar cultures grown at 20° C were used for agglutination As a rule h_γ agglutinins are not absorbed by live strain Cowan II bacteria grown on nutrient agar as found in the present study (cf Table 1)

Only strain Cowan I was able to exhaust the serum completely for agglutinins to nutrient agar cultures of the homologous strain The corresponding agglutinogen of strain Cowan I has preliminarily been designated Cowan factor-1, since the whole terminology has not yet been revised Absorption with strains 670 and 5687 (not included in the table) left weak antibodies to strain Cowan I, but it is not clear whether they are antibodies to antigen A (Jensen) which are poorly absorbed by these strains or residual antibodies to Cowan factor 1 None of the other type strains absorbed Cowan factor 1 agglutinins

In the next experiment serum Cowan I was absorbed with strain Cowan III by the standard technique, and thereafter portions of this serum were absorbed with each type strain with thrice the standard dose of bacteria

Table 2 shows that the immune serum is inadequately absorbed since

TABLE 1

Agglutinability of the Type Strains in Serum Cowan I Absorbed with Various Type Strains

Strain	Serum Cowan I absorbed with the standard dose of Cowan II bacteria						
	Not re absorbed	Re absorbed with thrice the standard dose of strains					
		Cowan II	1503	28	Wood 46	1015	Cowan I
1503	+++(250)	—	—	—	+	++	—
2253	+++(250)	—	—	—?	+	—	—
28	(+)	—	—	—	—	—	—
365	+++(100)	+(10)	—	+++	+++	++	—
3647	++(50)	—	—	—	+	+	—
F 21	+(50)	—	—	—	—	+	—
17 A	+++(500)	—	—	—	—	—	—
Wood 46	(+)	—	—	—	—	—	—
Cowan I	+++(500)	+++(250)	+	++	++	++	—
Cowan II	(+)	—	—	—	—	—	—
Cowan III	+++(25)	—	—	+	+++	++	—
670	++(50)	—	+	+	++	++	++
830	+(10)	—	—	(+)	++	—	—
5687	++(100)	—	+	+	++	++	+
6376	+(25)	—	—	—	++	—	—
3189 2035 and 1015	—						

Code (+) +, ++ and +++ Strength of agglutination
— No agglutination
Figures in brackets Resiprocals of agglutination titres

EXPERIMENTAL AND RESULTS

Serum Cowan I

Theoretically the following antibodies might be found in a Cowan I serum a_2, m, h_2, and l_2 (6, 7, 8) in addition to the new antibodies described in this article No a_2, m, h_2 or k_2 antibodies could be demonstrated in the immune serum used in the present investigations

Serum Cowan I was first absorbed with strain Cowan II, and portions of this serum were thereafter absorbed with each of our 18 type strains (Table 1)

It appears from Table 1 that strong agglutinins were left to most type [illegible] II or- [illegible] an II [illegible] with strains 28, Wood 46 and 1015 left agglutinins to a number of type strains as shown in Table 1 It has repeatedly been observed that strains 28 Wood 46, Cowan II, and 1015 fail to absorb agglutinins which are readily removed by most other strains In the present experiment agglutinins to strains other than Cowan I, 670, and 5687 were removed by all type strains except those mentioned above, and accordingly they represent most probably group agglutinins Strains 28 Wood 46, Cowan II and 1015 also agglutinated weakly or not at all after the first absorption with strain Cowan II (Table 1)

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[illegible]	+++(250)	—	—	—	+	++	—
[illegible]	+++(250)	—	—	—?	+	—	—
[illegible]	(+)	—	—	—	—	—	—
[illegible]	+++(100)	+(10)	—	+++	+++	++	—
[illegible]	++(50)	—	—	—	+	+	—
[illegible]	+(50)	—	—	—	—	+	—
[illegible]	+++(500)	—	—	—	—	—	—
W[illegible] 46	(+)	—	—	—	—	—	—
C[illegible] I	+++(500)	+++(250)	+	++	++	++	—
C[illegible] II	(+)	—	—	—	—	—	—
C[illegible] III	+++(25)	—	—	+	+++	++	—
[illegible]	++(50)	—	+	+	++	++	++
[illegible]	+(10)	—	—	(+)	++	—	—
[illegible]	++(100)	—	+	+	++	++	+
[illegible]	+(25)	—	—	—	++	—	—
[illegible] 2005 and 1015	—						

Code (+) + ++ and +++ Strength of agglutination
— No agglutination
Figures in brackets Resiprocals of agglutination titres

and h_3 antibodies, and mannitol-salt agar cultures of strain 365 (or 2253) for removal of h_2 antibodies

Serum Cowan II

Strain Cowan II has been found to possess the antigens h_2 and m (?) (7, 8) in addition to Cowan factor-2 and the new antigens published below Strain Cowan II has a very weak antigen A (Jensen)

Serum Cowan II was first absorbed with strain Cowan I, and portions of this serum were thereafter absorbed with each type strain using the same doses of bacteria as described in connection with serum Cowan I

TABLE 3

Agglutinability of the Type Strains in Serum Cowan II after Absorption with Various Type Strains

Strain	Serum Cowan II absorbed with the standard dose of strain Cowan I				
	Not re absorbed	Re absorbed with thrice the standard dose of strains			
		Cowan I	17 A	5687	Cowan II
Cowan II	+++(500)	+++(250)	+++	+++	—
1503	(+)	(+)	—	—	—
28	(+)	(+)	—	—	—
F 21	++(50)	—	—	—	—
17 A	++(500)	+(10)		—	—
Cowan I	(+)	—	—	—	—
The other type strains	—				

Code See Table 1

se
ag...
strains, and the corresponding antigen has been designated Cowan factor-3

Weak agglutinins were found to strains 1503, 28, 17 A and Cowan II They were absorbed by the same strains and probably also by strains 670 and 5687 After re absorption, however, the agglutinations were very weak and might well be overlooked The distribution of this antigen, designated Cowan factor 4, could therefore not be determined with certainty

Absorption of serum Cowan II with strain Cowan III and thereafter with each type strain did not lead to detection of new antigens Strong antibodies to Cowan factor 3 and weak antibodies to Cowan factors 2 and 4 were demonstrated

A *Cowan factor 3* serum can be prepared by absorption of serum Cowan II with strains Cowan I and 670 A suitable strain for selective absorption of Cowan factor-3 antibodies not being available a Cowan factor-4 serum could not be prepared

strain Cowan III still agglutinated The agglutinins to strains 365, 2095, and Cowan II appeared to be group agglutinins, since they were removed by re-absorption with all type strains except 1015, which left agglutinins to strain Cowan III

TABLE 2

Agglutinability of the Type Strains in Serum Cowan I after Absorption with Various Type Strains

Strains	Serum Cowan I absorbed with the standard dose of strain Cowan III					
	Not re absorbed	Re absorbed with thrice the standard dose of strains				
		Cowan III	*Cowan II*	*Cowan I*	*1015*	*670*
1503	+++(250)	+++(250)	—	—	+++	—
2253	(+)	(+)	—	—	—	—
28	+++(250)	++(250)	—	—	+++	—
365	++(50)	—	—	—	—	—
17 A	+++(500)	+++(250)	—	—	+++	—
2095	(+)	—	—	—	—	—
Cowan I	+++(500)	+++(250)	+	—	—	—
Cowan II	+++(1000)	+++(1000)	—	—	+++	—
Cowan III	+(50)	—	—	—	+	—
670	++(50)	++(10)	++	++	++	—
5687	++(100)	+(10)	++	++	++	—
The other type strains	—					

Code See Table 1

As was expected h_- agglutinins were left after re-absorption with live nutrient agar cultures of the homologous strain Re-absorption with strain Cowan II left in addition antibodies to Cowan factor-1

The strong agglutination of strains 1503, 28, 17 A, Cowan I, and Cowan II was abolished on absorption with the same strains and also by strains 670 and 5687 The latter strains exhausted the serum and seem accordingly to possess the Cowan factor-1 The new strong agglutinogen has been designated Cowan factor-2

A factor serum to Cowan factor-1 can be prepared by absorption of serum Cowan I with live nutrient agar cultures of strain 365 and mannitol-salt agar cultures of strain 1503 The Cowan factor-1 agglutinins of the present serum were weak As mentioned below under "Discussion" the Cowan factor-1 antigen is most probably identical to *Hofstad*'s 263-1 antigen (12, 13) If so, a potent serum can be prepared by his method

Since we at present have no strain for selective absorption of Cowan factor 1 antibodies, a pure Cowan factor-2 serum cannot be prepared Weak Cowan factor-2 antibodies have also been found in 17 A and 1503 immune sera, but they were too weak for preparation of a factor serum, and our set of type strains is inadequate for absorption

A Cowan factor-1-2 can be prepared by absorption of serum Cowan I with nutrient agar cultures of strain 365, which remove possible a_2, *m*,

at 20° C agglutinated more strongly than the same cultures grown at 37° C and ordinary nutrient agar cultures (Table 4), the former type of cultures was selected for the re-absorption experiments None of the strains exhausted the serum Strains 28, 17 A, and Cowan II reduced the 670 1 agglutinin titre, but it cannot be excluded that such a weak absorption is unspecific

TABLE 4

Agglutination with Various Cultures of Strain 670 in 670 1 Factor Serum

Strain	Agglutinability in 670-1 factor serum		
	Nutrient agar cultures	Mannitol-salt agar cultures at 37° C	Mannitol salt agar cultures at 20° C
670	+	+	+++

Code See Table 1
The 670 1 factor serum was prepared by absorption of serum 670 with strain 5687

Serum 5687

Strain 5687 has the ability to absorb most of the known staphylococcal agglutinins, although the strain agglutinates in very few of the factor sera It is not clear whether or not the absorption is unspecific, and aside from the h_2 antigen the antigenic make-up of the strain is not known with certainty

The antibodies of serum 5687 were examined by absorption of portions of the serum with each of the type strains as with serum 670 Four agglutination patterns were obtained (Table 5), indicating the presence of at least 2 different antibodies Agglutination with mannitol-salt agar cultures revealed h_2 agglutinins (in Table 5 after absorption with strains Cowan I and Cowan II) Absorption with strain 670 left agglutinins to strains Cowan II and 5687 This new antigen has been designated 5687 1

TABLE 5

Agglutinability of the Type Strains in Serum 5687 after Absorption with Various Type Strains

Strain	Serum 5687 absorbed with strains			
	Cowan I	Cowan II	670	5687
Cowan II	++	−	++	−
670	+++	++	−	−
5687	+++	++	(+)	−
The other type strain	−	−	−	−

Code See Table 1

A potent h_2 serum was prepared by absorption of serum 5687 with strains 3647 and 17 A (or Cowan II)

A 5687-1 serum was prepared by absorption of serum 5687 with strains 3647 and 670 The agglutinability of the type strains in this

Originally a fifth Cowan factor was included, but the agglutination reactions were very weak. Strong agglutinins to apparently the same antigen were found in strain 5687 immune serum and will be described below.

A strong precipitation line in agar has been demonstrated corresponding to the Cowan factor-3 antigen and its antibody (cf. (17)). Like the agglutinins the precipitins were not absorbed by the other type strains. When recently these results were re-examined, neither the Cowan factor-3 agglutination nor the precipitation line could be demonstrated. Another Cowan II serum and other samples of the strain were examined but results were negative.

The Cowan II type strain 6128 showed almost identical agglutination patterns to strain 8531, but on absorption of serum 8531 with strain 6128 weak agglutinins were left to the homologous strain. Distinctly different precipitation patterns in agar were demonstrable between the two strains.

Serum Cowan III

Strain Cowan III possesses all the *a*, *b*, and *c* antigens except b_3 (6).

Serum Cowan III was examined as the two other Cowan sera, being first absorbed with one of the other Cowan strains and thereafter with each type strain. Agglutinins to most of the *a*, *b*, and *c* antigens were demonstrated in addition to *n* agglutinins. No new agglutinins were detected.

Serum 670

Strain 670 has been found to possess the following antigens: a_2, a_3, b_3, h_1, h_2, Cowan factors-1, -2, and -4, and 5687-1 (cf. (6), (7) and below).

To examine its antibody composition portions of serum 670 were absorbed with each type strain with the growth of one Roux bottle added to one ml of serum diluted 1 in 10, i.e. thrice the standard dose of bacteria. No h_1 antibodies were detected since agglutination was not obtained with strain 17 A after absorption with strain 5687, which possesses all the antigens of strain 670 except h_1. The absorption left, however, residual agglutinins to strain 670. This new antigen has been designated 670-1.

In addition serum 670 contained rather strong h_2 antibodies. Absorption with strains 28, 17 A, Wood 46 and 1015 left agglutinins to group antigens.

The distribution of the 670-1 antigen among the type strains could not be determined from the absorption experiments above, since the h_2 agglutination of strain 670 masked the 670-1 agglutination. It was therefore necessary first to absorb serum 670 with strain 5687 to remove h_2 agglutinins, and thereafter portions of this serum were re-absorbed with each type strain. As it was found that mannitol salt agar cultures grown

designated 6376-1 The reactivity of the new agglutinogen was too weak to be subjected to further studies with regard to its distribution among the type strains

Serum 830

Strain 830 possesses all the *a*, *b*, and *c* antigens except a_1 and c_1 (6) Absorption of the serum with the type strains revealed a_1, a_5, and b_1 antibodies The serum contained very strong a_5 antibodies and will provide a potent a_5 factor serum when suitable strains for absorption have been found

No new agglutinogens were detected

Serum Wood 46

Strain Wood 46 has the antigens a_1 and t_1 (5, 8) Absorption experiments revealed no antibodies to known agglutinogens other than t_1, and no antibodies to antigen A (Jensen) As reported in (3) the serum possessed very strong antibodies to polysaccharide A After absorption with the polysaccharide strong agglutinins to some type strains were found (4) These agglutinins could not be demonstrated in sera absorbed with whole bacteria, and they represent most probably antibodies to an unknown group antigen

Distribution of the New Antigens among the Type Strains (Table 7)

The distribution will be discussed in the next article and correlated with the other antigens and phage types

TABLE 7

Distribution of the Antigens Among the Type Strains

Strain	Antigen					
1503		Cow f -2		Cow f -4		
28		Cow f 2		Cow f -4	(670-1)?	
17 A		Cow f 2		Cow f 4	(670-1)?	5687 1
Cowan I	Cow f 1	Cow f 2				5687 1
Cowan II		Cow f -2	Cow f -3	Cow f 4	(670-1)?	5687-1
670	(Cow f 1)	(Cow f -2)		(Cow f -4)	670-1	5687-1
5687	(Cow f 1)	(Cow f 2)		(Cow f 4)		5687 1

Antigens listed in brackets The agglutinogen could only be demonstrated by absorption

Preparation of Individual Factor Sera

Cowan factor 1 serum Serum Cowan I absorbed with nutrient agar strain 365 bacteria and mannitol salt agar strain 1503 bacteria (Cf also *Hofstad* (12))

Cowan factor 1-2 serum Serum Cowan I absorbed with nutrient agar and mannitol-salt agar cultures of strain 365

serum was tested with ordinary nutrient agar cultures as well as with mannitol-salt agar cultures grown at 37° C and 20° C (Table 6)

TABLE 6

Agglutinability of Various Cultures of the Type Strains in 5687-1 Factor Serum

Strain	Agglutinability in 5687 1 factor serum		
	Nutrient agar cultures	Mannitol salt agar, cultrs at 37 C	Mannitol salt agar cultrs at 20 C
17 A	—	+++	+
Cowan I	—	++	+
Cowan II	++(+)	++	+
670	—	(+)	(+)
830	—	—	+(+)
5687	(+)	++	—
The other type strains	—	—	—

Code: See Table 1

The 5687-1 factor serum was prepared by absorption of serum 5687 with strains 3647 and 670

It appears from Table 6 that the 5687-1 antigen, like the h_2 antigen, was partly or completely blocked in some strains cultivated on nutrient agar, but unlike the h_2 antigen the agglutinability of cultures grown at 37° C on mannitol-salt agar was greater than when grown at 20° C The 5687-1 factor serum was absorbed with mannitol-salt agar cultures of the type strains grown at 37° C The absorptions confirmed the presence of the 5687-1 antigen in strains 17 A, Cowan I, Cowan II, 670, and 5687 Absorption of the serum with mannitol-salt agar cultures of strain 830 grown at 20° C neutralized only the homologous reaction It can therefore be necessary to absorb the serum with mannitol-salt agar cultures of strain 830 grown at 20° C in addition when a h_2 or a 5687-1 factor serum is prepared from serum 5687

The agglutinogen of strain 830 has not been examined further

Serum 1015

Strain 1015 possesses a strong c_1 agglutinogen (6) and weak group antigens

Serum 1015 was absorbed with each of the type strains, but the agglutination patterns obtained revealed no new agglutinogens After absorption with strain 2095 a potent factor c_1 serum was obtained

Serum 6376

Strain 6376 has the antigens a_1, a_2, b_1, b_2, b_3, ac_1, and c_1 (6)

Serum 6376 was absorbed with each of the type strains and like serum 1015 it contained strong c_1 antibodies, but no antibodies to the other antigens mentioned above Absorption with strains 2095 and 1015 left weak agglutinins to the homologous strain The agglutinogen was

other investigators is not possible It is, however, evident that the 3 Cowan strains have widely different immunological properties, both with regard to type and group antigens and agar precipitation patterns C : [illegible] identical to *Hofstad*'s (12) 263-1 antigen Str [illegible] this [illegible] factor serum The antigen seemed to be specific to phage group [illegible]

Cowan factor-2 was the major type antigen of strain Cowan I in our immune serum It is interesting to note that strains possessing this antigen also agglutinate in h_2 factor serum, but in the latter serum they often require the use of mannitol-salt agar cultures for agglutination [illegible] the Cowan factor-3 agglutination and precipitation is not [illegible]

body was produced in the other rabbit immunized Strong [illegible] Cowan II have been demonstrated in serum 5687, *Hofstad* (12) found probably the same antibody also in serum Cowan II, and it cannot be excluded that the corresponding antigen (5687 1) is identical to Cowan factor 3

As mentioned above antigen 5687-1 is probably identical to *Hofstad*'s antigen 263 2 antigen, which was demonstrated in 40 of 45 phage group I strains using 18 hours nutrient agar cultures for agglutination

The original Cowan II strain 8531 and Dr *Christie's* strain 6128 showed almost identical agglutinogens Possible specific antigens of strain 6128, however have not been searched for, since no serum was produced against this strain *Pillet* (18) reports that he has demonstrated a specific agglutinogen in strain 8531

SUMMARY

The antibody composition of immune sera against strain Wood 46, the Cowan strains, and the new type strains has been examined Seven new antigenic factors have been described

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Cowan factor-3 serum: Serum Cowan II absorbed with strains Cowan I and 670.

670-1 serum: Serum 670 absorbed with strains 1503 and 5687.

h_2 serum: Absorption of serum 5687 with strains 3647 and 17 A (or Cowan II).

5687-1 serum: Absorption of serum 5687 with strains 3647 and 670

6376-1 serum: Absorption of serum 6376 with strains 2095 and 1015

TABLE 8

Agglutinability of the Cowan Strains after Absorption of the Cowan Sera with the Two Heterologous Cowan Strains

Immune serum	Absorbed with Cowan strain	Agglutinability with Cowan strain		
		I	II	III
Cowan I	II	+++	—	—
	III	+++	+++	—
Cowan II	I	—	+++	—
	III	++	+++	—
Cowan III	I	—	—	+++
	II	++	—	+++

Code See Table 1

DISCUSSION

The immune sera against the 3 Cowan type strains differ considerably in antibody composition Serum Cowan I contains, after absorption with strain Cowan II, strong antibodies to one or more group antigens (antigen A (Jensen)?), and after absorption with strain Cowan III strong Cowan factor-2 antibodies Serum Cowan II will contain Cowan factor-3 or/and -4 antibodies after absorption with the two other Cowan strains or possibly also 5687-1 antibodies Serum Cowan III will possess strong *a*, *b*, *c*, and *n* antibodies after absorption with the other Cowan strains, and after absorption with strain Cowan II in addition group agglutinins The results have been summarized in Table 8, and they agree completely with *Stern & Elek's* (20) results summarized in their Table 2 The content of group agglutinins after absorption with strain Cowan II depends on the dose of bacteria used for absorption as evident from the absorption and re-absorption experiments with increasing doses of bacteria, and from *Stern & Elek s* quantitative absorption technique (20)

It appears from the present investigations that the Cowan sera, also after absorption may contain a complex of antibodies, and the antibody composition is likely to vary according to the batch of immune serum employed As a consequence of this and of possible differences in technique, a more precise correlation of the present findings with those of

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The University Institute of General Pathology, Copenhagen
(Chief Professor K A Jensen, M D)

A STUDY ON PROBLEMS OF RESISTANCE

2 *Investigations into the Emergence of Resistance of Tubercle Bacilli to Isoniazid*

By

K A JENSEN and INGER KIER

Received 4 x 63

In a previous paper (1) on the prophylactic treatment of tuberculosis with isoniazid (INH) mention was made of studies concerning the mechanism of the emergence of INH-resistance Preliminary investigations in which *Esch coli* and streptomycin had been selected as model had shown that resistance could emerge by spontaneous mutation independent of streptomycin or by mutation that was demonstrated by cultivation of *Esch coli* on a medium to which streptomycin has been added In spite of repeated replication experiments in our Institute, however, we have only twice succeeded in obtaining a culture of spontaneously streptomycin resistant mutants of the coli bacilli, which might imply that they are extremely rare There is, however, one factor which suggests that they are more common than previously assumed It was found that the two spontaneous mutants cultivated by us multiplied more slowly than did the mother strain on the medium without streptomycin

This affords an explanation of the difficulty experienced by the writers as well as by others in obtaining pure cultures of these spontaneous mutants in replication experiments It is, in fact, surprising that this is possible at all, and here we also have an explanation of why all coli bacteria are not resistant to streptomycin

With a view to ascertaining the actual incidence of resistant mutants, the selection principle was employed A non streptomycin-resistant coli bacillus was cultivated on agar plates to which streptomycin had been added in the following concentrations 5, 10, 20, and 40 μg per ml of medium, in order to eliminate the streptomycin-sensitive bacilli Growth of numerous colonies was obtained on the medium with 5 μg of streptomycin while only a few colonies appeared on the medium with 40 μg Sensitivity determinations of the colonies cultivated for 24 hours on the 5 μg medium showed no notable increase in the resistance

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After cultivation for 48 hours at 37° C the cultures were left standing at room temperature, and the following interesting observation was made. After a few days a clear segment-shaped outgrowth appeared along the marginal zone of the colonies and later eugonic secondary colonies emerged from these outgrowths.

Resistance determinations on cultures from the mother colony, the clear segments, and the eugenic segments gave the following results: Only a slight increase was seen in the cultures from the mother colony, considerably increased resistance in cultures from the clear segments, while cultures from the eugonic segments showed excellent growth on the medium with 40 µg of streptomycin. It is difficult to ascertain whether this *last-mentioned mutation to streptomycin resistant coli bacilli is due to adaptation or to spontaneous mutation.* There is reason to believe that with the above mentioned procedure it has been practicable to "capture" the more slowly growing resistant mutants, since all the resistant strains cultivated from the segments grew more slowly on agar without addition of streptomycin than did the mother culture—in analogy with the two spontaneous mutants cultivated in the replication experiments.

After this experiment we investigated whether the same condition applied to the emergence of resistance of the tubercle bacillus to INH.

EXPERIMENTAL SERIES 1

Technique

Here our old standard strain L 5—cultivated from a patient in 1935—was employed. From a young but well developed culture on modified Lowensteins medium (I.U.T.M.) a suspension containing 1 mg of tubercle bacilli per ml was prepared (about 50 million germinative tubercle bacilli per ml). Tubes containing 0, 0.02, 0.04, 0.08 and 0.32 µg of INH per ml of the medium were inoculated with 0.1 ml of the undiluted suspension and with dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} respectively. The tubes were left in a horizontal position for 24 hours until the fluid had been absorbed by the medium and then placed in incubator at 37–38° C. After incubation for 3 and 4 weeks the number of colonies was determined *in the* tubes that contained from 3 to 50 colonies.

From well isolated colonies on the media containing 0.02, 0.04, 0.08 and 0.32 µg of INH new suspensions were prepared and inoculated as described above. This procedure was repeated until the culture had been through up to 5 passages on 0.02 µg of INH. In the case of the higher concentrations one passage was sufficient for the emergence of resistance.

The result is seen in Table 1.

The second column shows the origin of the colony and the third column the number of germinative tubercle bacilli in millions per ml in the suspension used for the inoculation. The figures in the next columns indicate the number of colonies per 1000 colonies in the tubes without INH.

From the table it is apparent that even after 5 passages of L5 on the medium with 0.02 µg of INH per ml there had been no definite increase in the resistance to the drug. During these five passages the tubercle bacilli have been cultivated for about six months on the medium con-

TABLE 1

			0 02 γ	0 04 γ	0 08 γ	0.32 γ
				Original culture		
E5 Col 1		30	0 1	0 001	0 0001	<0 0001
E5 Col 2		18	0 4	0 0006	0 0002	<0 0002
E5 Col 7		25	0 3	0 0009	0 0001	<0 0001
E5 Col 8		67	0 7	0 01	0 0001	<0 00005
				1st passage on 0 02 γ INH		
Col 3		22	0 5	0 001	0 0001	<0 0001
Col 5		29	0 1	<0 0003	<0 00003	<0 0001
				2nd passage on 0 02 γ INH		
Col 17	(3)	50	–	0 01	0 001	0 001
Col 54	(5)	19	–	0 03	0 002	0 0007
Col 55	(5)	17	8 4	0 02	0 003	–
Col 56	(5)	22	3 2	0 005	0 001	–
Col 57	(5)	26	10 0	0 005	0 001	
				3rd passage on 0 02 γ INH		
Col 60	(55)	130	5 4	0 003	0 002	0 00005
Col 61	(55)	49	10 8	0 002	0 0008	0 0004
Col 62	(57)	60	1 3	0 003	0 002	0 0002
Col 63	(57)	36	9 2	0 004	0 0003	<0 0001
Col 65	(56)	70	1 3	0 003	0 001	0 0006
Col 66	(56)	33	5 5	0 004	0 0001	0 0001
				4th passage on 0 02 γ INH		
Col 67	(60)	33	5 8	0 008	0 004	0 001
Col 68	(60)	27	1 9	0 004	0 002	0 002
Col 69	(62)	50	5 8	0 08	0 003	0 002
Col 70	(62)	32	2 5	0 0009	0 0009	0 0002
Col 71	(61)	81	0 1	0 0003	0 00008	0 00004
Col 72	(61)	14	0 9	0 006	0 004	0 0002
Col 73	(62)	8	0 6	0 006	0 002	0 0009
Col 74	(62)	21	0 3	0 005	0 002	0 0008
Col 75	(63)	7	0 4	0 003	0 001	0 0004
Col 76	(63)	18	0 8	0 008	0 003	0 0004
Col 78	(65)	21	0 7	0 02	0 007	0 003
Col 79	(65)	90	0 04	0 0001	0 0001	0 00003
Col 82	(65)	24	5 0	0 008	0 003	0 0005
Col 83	(65)	15	2 7	0 02	0 0007	0 0007
				5th passage on 0 02 γ INH		
Col 84	(65)	20	10 5	0 03	0 01	0 001
Col 89	(70)	47	1 0	0 005	0 003	0 0009
Col 90	(78)	26	1 1	0 008	0 003	0 0009
Col 91	(78)	14	0 4	0 03	0 009	<0 0002
				E5, control at the end of the experiment		
E5		33	5 7	0 003	0 001	0 0002

taining 0 02 μg INH From this it is apparent that the development of resistance is not due to training

Something very different applies to the colonies which in the first culture grew on 0 04, 0 08, and 0 32 μg of INH respectively

Nineteen colonies which in the first culture had grown on medium containing 0 04 μg INH were resistance-determined

Of these 19 colonies 10 were resistant to 1 28 μg of INH, 3 were restant to 0 32 μg, one to 0 08 μg, and 3 to 0 04 μg Only two colonies had not developed resistance

Eleven colonies cultivated from the medium containing 0 08 μg INH were also investigated Nine proved to be resistant to 1 28 and 2 to 0 08 μg INH

Two colonies from the medium with 0 32 μg INH were tested, and both proved resistant to 1 28 μg of INH

The explanation of this obvious difference between the emergence of resistance on the medium with 0 02 μg INH and on media containing 0 04 and higher concentrations of INH is presumably as follows The 0 02 μg medium yields well-isolated colonies upon inoculation with 0 1 ml of suspension diluted about 100,000 times, while the 0 04 μg medium usually has to be inoculated with 0 1 ml of the suspension diluted about 100 times Owing to this much larger inoculum, the possibility of selection of resistant mutants is far greater on the 0 04 μg medium, and even greater on the 0 08 and 0 32 media which have to be inoculated with undiluted suspension This also implies that the resistance emerges by selection of those tubercle bacilli that have the property—latent or manifest—of becoming resistant to INH The fact that an increase in resistance of up to 64 times may occur after one passage alone also speaks against training

EXPERIMENTAL SERIES 2

After having ascertained—in experimental series 1—that the emergence of resistance to INH probably depended upon the size of the inoculum, similar experiments were performed but with the modification that instead of preparing the suspension of tubercle bacilli from a single, *well-isolated colony, suspensions were used that had been produced* from scrapings of the entire culture from tubes containing 0 02 μg of INH inoculated with 0 1 ml of undiluted suspension

In addition, we investigated whether tubercle bacilli cultivated from different patients possessed a different ability to develop resistance when cultivated on the medium containing 0 02 μg of INH

The following 21 cultures were examined

(a) cultures from patients before the discovery of INH namely F5 SS26 SS1, SS56, SS2 SSK, and S532

(b) cultures from patients who had not previously been treated with INH 10252 10443, 10256 10257, 10266 10442 10454 and 10457

(c) cultures from patients treated with combined therapy, including INH
10368 and 10243 treated for 1 month with INH + PAS
10476 treated for 6 weeks with INH + PAS.
10458 treated for 3 months with INH + PAS + streptomycin
10487 and 10488 treated for 5 months with INH + PAS + streptomycin

Owing to lack of space the results obtained from this large series of experiments are not recorded in their entirety At the conclusion of the experiment, the strains were divided into four groups Group 1 comprises strains in which resistance *emerged rapidly, up to* 0.32 μg INH, group 2 comprises those strains in which the emergence of resistance was somewhat slower, but also up to 0.32 μg INH, group 3, strains in which *resistance developed slowly, while Group 4 had only one strain* in which the increase in resistance was slight

In Table 2 *one strain of each group is recorded The symbols are the* same as in Table 1

TABLE 2

		0.02γ	0.04γ	0.08γ	0.32γ
				Group 1	
10443	37	0.28	0.002	0.0003	0.00007
1st passage	70	9.53	0.47	0.67	0.09
2nd passage	37	427.0	82.0	95.0	4.6
				Group 2	
10457	30	10.7	0.04	0.0009	<0.0001
1st passage	50	47.0	3.8	0.4	0.002
2nd passage	30	322.0	21.0	2.1	0.02
3rd passage	29	383.0	17.0	5.7	0.06
4th passage	27	137.0	47.0	5.4	0.1
5th passage	33	514.0	90.0	6.0	0.4
6th passage	22	1000.0	217.0	40.0	0.4
				Group 3	
10487	113	13.9	0.01	0.0003	<0.0005
1st passage	50	51.0	0.8	0.19	0.0005
2nd passage	22	106.0	32.0	7.0	0.001
3rd passage	20	417.0	134.0	45.0	0.001
4th passage	53	251.0	82.0	25.0	0.007
5th passage	40	500.0	54.0	13.0	0.008
6th passage	37	459.0	108.0	22.0	0.05
				Group 4	
10243	40	12.0	0.1	0.0006	<0.00008
1st passage	33	190.0	0.4	0.006	0.0004
2nd passage	50	174.0	0.7	0.003	0.00006
3rd passage	30	633.0	2.0	0.01	0.0002
4th passage	24	570.0	11.0	0.02	0.002
5th passage	21	603.0	8.3	0.03	0.005
6th passage	53	619.0	1.6	0.01	0.0008

TABLE 3

	0 02 γ	0 04 γ	0 08 γ	0 32 γ
			Group 1	
Original culture	3 45	0 082	0 0062	0 0017
1st passage	90 2	33 7	5 54	0 09
2nd passage	338 5	77 7	62 2	2 52
			Group 2	
Original culture	19 5	0 011	0 0014	0 0004
1st passage	93 9	2 44	0 25	0 031
2nd passage	377 0	97 1	12 1	0 15
3rd passage	419 0	62 2	13 6	0 43
4th passage	466 0	185 0	114 0	2 18
5th passage	589 0	135 0	16 4	0 25
6th passage	845 0	237 0	32 2	0 46
			Group 3	
Original culture	63 6	0 16	0 0013	0 00013
1st passage	205 0	2 72	0 11	0 00051
2nd passage	180 0	16 4	2 55	0 00054
3rd passage	477 0	60 3	15 6	0 0014
4th passage	551 0	74 4	10 52	0 0028
5th passage	689 0	170 0	12 5	0 012
6th passage	630 0	125 0	8 0	0 013
			Group 4	
Original culture	11 8	0 012	0 0006	0
1st passage	190 0	0 37	0 006	0 0004
2nd passage	174 0	0 73	0 003	0 00006
3rd passage	633 0	2 0	0 012	0 0002
4th passage	570 0	11 2	0 021	0 0018
5th passage	603 0	8 3	0 03	0 005
6th passage	619 0	1 63	0 013	0 0008
			E5 Experimental series 1	
Original culture	1 4	0 0031	0 0003	0 00004
1st passage	0 3	0 0005	0 00005	0
2nd passage	7 1	0 014	0 0016	0 0009
3rd passage	3 9	0 0032	0 001	0 0002
4th passage	1 96	0 012	0 0023	0 0087
5th passage	3 25	0 012	0 0063	0 0007

In Table 3 the average number of colonies of the strains in the various groups is calculated The number of colonies is given per thousand of the number of germinative tubercle bacilli in the suspension For the sake of comparison the mean values of the various passages of strain E5, where bacterial suspensions of an isolated colony were prepared (Table 1), have been recorded at the foot of Table 3

The curves representing the four groups are plotted on Fig 1

From Tables 2 and 3 it is seen that upon passage of tubercle bacilli including E5 on medium containing 0 02 μg of INH/ml there was a con

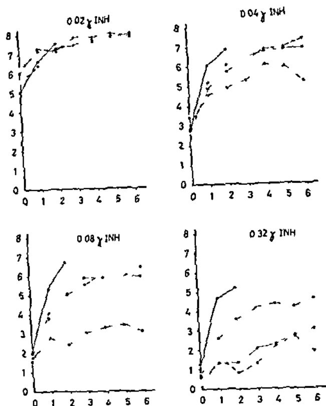


Fig 1

In Table 2 tl
This f gure s
Log 8 corre

Group 1 ●———————● Group 2 ●— — — — —●
Group 3 ● ● Group 4 ●— — — — —●

siderable increase in resistance unlike the findings in experimental series 1 The only difference between these two experimental series is that in the first one inoculations were made from a suspension prepared from a single colony taken from tubes seeded with 10^{-4}–10^{-6}, while in the second series we used a suspension prepared from scrapings of a culture seeded with 0 1 ml of undiluted culture suspension As the inoculum was the same in both experimental series, the increase in resistance in series 2 can be due only to the greater possibility of selection of the tubercle bacilli capable of developing resistance

From Tables 2 and 3 it will also be seen that the 21 strains of tubercle bacilli cultivated from patients show a pronounced difference in ability to develop resistance on passage on 0 02 μg INH This is also apparent from Fig 1 where the results from Table 3 are plotted as curves

This difference observed *in vitro* appears to be confirmed by the following investigations on the resistance emergence of tubercle bacilli cultivated from patients suffering from cavernous pulmonary tuberculosis and treated with INH alone

Incipient emergence of resistance to INH was observed after 14, 17, 18, 22, 24, 29, 30, 37, 39, 41, 41, 42, 43, 46, 47, 48, 62, 63, 63, 65, 67, 75, 78, and 93 days' treatment respectively, while in two cases resistance was not seen after 92 and 93 days' treatment Total resistance to 0 32 μg of INH emerged after 37, 40, 45, 47, 48, 57, 58, 60, 63, 66, 67, 69, 70, 75, 76, 78, 79, 87, 91, 93, 103, and 147 days' treatment with INH alone, while in three cases total resistance to 0 32 μg INH had not emerged after 92, 93, and 99 days respectively. This corresponds to about sixteen times the resistance of the standard strain E5

This also shows that in the treatment of cavernous pulmonary tuberculosis with INH alone, there is a marked variation in the rate at which resistance emerges

It must be admitted that there are other factors that influence the rate at which resistance emerges during treatment with INH, but these factors alone cannot give rise to this marked variation

Thus there can be hardly any doubt that the ability of the strains of tubercle bacilli to develop resistance is markedly variable, and those strains that easily develop resistance will, in the long run, be selected in countries where patients are treated with INH alone (cf the selection of penicillinase-producing staphylococci)

Unfortunately, however, with our usual methods for resistance determination it is impracticable at the beginning of treatment to determine the ability of the tubercle bacilli to develop resistance

There is still another factor that cannot be elucidated by means of the methods usually employed in resistance determination In our Institute the direct sensitivity test is used for all sputa in which tubercle bacilli are demonstrated microscopically In the case of sputa that are negative on microscopy, and in cases where the direct sensitivity test shows growth of only 50–100 colonies on medium without INH, indirect resistance determinations are made, *i e*, resistance determination test on scrapings of the colonies on the control tubes

In the direct resistant test the size of the inoculum varies from about 1/20 1/300,000 of the inoculum employed in experimental series 1 and 2 In the secondary resistance determination test the inoculum employed is about 1/200 of that used in experimental series 1 and 2 Moreover, in the secondary resistance determination, suspensions are prepared from the control tubes which usually contain but a few colonies—often only one or two In this way, the secondary resistance determination will resemble experimental series 1, in which inoculations were made from a single colony

Thus, in primary resistance determination there must be from 10–30,000 times as many resistant mutants before they can be detected

... method and in the secondary resistance determination ...

resistance emergence will in many cases not be detected ... the values reported in the literature for primary INH resistance must be minimal values

In a previous paper this resistance has been called "latent" By means of our usual resistance determination methods it is not possible to ascertain how far the latent resistance has advanced, and how near it is to the stage at which it will be observed in a routine resistance determination There is hardly any doubt, therefore, that some error must be present in the data available regarding resistance determination tests in treatment with INH alone and with INH combined with other drugs

In the present material of 21 strains, the emergence of resistance is distributed over the four groups showing the various degrees of rapidity of resistance development (see Table 4)

TABLE 4

	Group 1		Group 2		Group 3	
	Number of strains	Duration of treatment in months	Number of strains	Duration of treatment in months	Number of strains	Duration of treatment in months
Cultivated before the discovery of INH	3		2		2	
Cultivated after the discovery of INH but not treated	2		4		2	
Cultivated after the discovery of INH combined therapy	1	1 5	1	1	1	1
	1	3	1	3	1	5

The material is small but from the table it is evident that the capacity for increased resistance is associated with the individual strains Moreover, it is interesting to note that 1–5 months' treatment with INH combined with other drugs has not altered the latent resistance

It would be of interest in a larger material to investigate how this capacity changes under combined therapy throughout the treatment Such a procedure would, however, be encumbered with the drawback that after 3 5 months' combined therapy it is not possible to cultivate tubercle bacilli from the patients

SUMMARY

Our investigations into the emergence of resistance of the tubercle bacillus to INH showed that this is due to the selection of INH-resistant mutants, not to training The demonstration of resistant mutants is difficult as they grow more slowly than the mother culture Such mutants have, therefore, been considered as extremely rare By means of the selection principle, however, we have been able to demonstrate that they occur in greater numbers than has previously been assumed

On examination of 21 strains of tubercle bacilli cultivated from patients with pulmonary tuberculosis, a marked difference in their capacity to develop resistance to INH was demonstrated We have called it "latent resistance"

This difference in latent resistance demonstrated *in vitro* corresponds to our experience concerning the treatment with INH alone of patients suffering from cavernous pulmonary tuberculosis Our present methods for testing the sensitivity of tubercle bacilli furnish no information regarding latent resistance, nor do they throw any light worth mention on how far the resistance has developed at that stage of the treatment when no increase in the resistance is demonstrated by means of the usual methods now employed

There is hardly any doubt, therefore, that the reported incidences of primarily INH-resistant cultures in the various countries are minimum values

Since the resistant mutants are more common than has previously been assumed, the possibility of selection during treatment with INH alone does not require the presence of a large population of tubercle bacilli as in cavernous pulmonary tuberculosis, but may also occur in the treatment of primary tuberculosis If the effect of our most important drug against tuberculosis is to be preserved as long as possible, the inadvisability of using INH alone in the treatment of tuberculosis must be strongly emphasized

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The Enterovirus Department, Statens Seruminstitut, Copenhagen, Denmark

INTRATYPIC SERODIFFERENTIATION TESTS ON POLIO TYPE 1 VIRUS STRAINS ISOLATED BEFORE AND AFTER VACCINATION WITH ATTENUATED SABIN TYPE 1 POLIO VACCINE

By

FIN HAHNEMANN, KALD SIBONI and
ANNELISE GODTFREDSEN

Received 19 xi 63

The problems of intratypic serological differentiation between wild, virulent strains and the progeny of attenuated vaccine strains isolated [illegible]

virus strains In previous publications from this laboratory (7, 8) the production of specific antisera and the employment of plaque technic as well as neutralization tests in roller tubes for serodifferentiation have been described

The present paper describes studies on serodifferentiation between strains of poliovirus type 1 isolated in Denmark before and after the vaccination campaign in April-May 1963, with Sabin type 1 attenuated vaccine Studies on T markers (9, 10) and d markers (11–13) on strains isolated from a patient with paralytic polio are also included

MATERIALS AND METHODS

Virus strains

The following strains of poliovirus type 1 were used

Mahoney

LSc 2ab

These 2 strains have kindly been supplied by Dr *Albert B Sabin* and have been carried for respectively 2 and 1 tissue culture passages in this laboratory

Sabin Connaught 109–2 and 110 3 two different lots of live type 1 polio vaccine (Sabin s strain LSc 2ab) manufactured by Connaught Laboratories in Toronto Canada

Brunhilde kindly supplied by Dr *A Svedmyr*

Chat Koprowski s attenuated type 1 strain, kindly supplied by Dr *M Bottiger* and carried in 4 additional monkey kidney tissue culture passages in our laboratory

A total of 49 polio type 1 virus strains isolated from human faeces in monkey kidney tissue cultures and stored at —20° C for periods varying from a few days

Some of the data in this paper have been presented at the 9' European Symposium of Poliomyelitis, Stockholm Sept 1963

to 7 years They were typed and titrated and used in their first tissue culture passage

Antisera The antisera were produced by inoculation of rabbits with virus without adjuvans 7 ml subcutaneously, 1 ml intradermally and 2 ml intravenously on the same day and bleeding by heart puncture after one week The sera were inactivated at 56° C for 30 min Six selected antisera against type 1 poliovirus strains described in the preceding paper (2) were employed 1) Sera Nos 601, 604 and 640 all produced by immunization with the Mahoney strain and in the following called anti Mahoney sera 2) Sera nos 623 610 and 628 produced by immunization with LSc 2ab 10077 II and 10077 VIII respectively but in the following simply referred to as anti LSc 2ab sera The two last strains were isolated from a 6 year old healthy boy 4 days and 43 days after he had been fed the Sabin type 1 vaccine They have been found to be serologically closely related to the LSc 2ab strain (2)

In order to economize with the sera a 1 16 dilution of the sera in 1 per cent gelatine was prepared and stored at —20° C During a 3 months storage period no decline in serum titres or in specificity of the sera was observed

Tissue cultures Trypsinized monkey kidney cultures of Cynomolgus Rhesus and Cercopitheeus monkeys were used at random Roller tube cultures were made by seeding of 100 × 15 mm tubes with 200 000 cells in Hank s BSS with 0 5 per cent lactalbumin hydrolysate and 2 per cent calf serum They were used on the 4 day after the medium had been changed to 1 8 ml of medium 199 without serum In addition, monolayer cultures were grown in glass bottles (Brockway bottles) having a flat side with an area of 32 0 cm^2 They were seeded with 4 × 10^6 versenated 1 passage cells in 20 ml Hanks' BSS with 0 5 per cent lactalbumin hydrolysate and 2 per cent calf serum They were used when confluent usually on the 4 day

Virus titrations and neutralization tests For virus titrations 10 fold dilutions were made in saline and 5 roller tubes were used per dilution They were inoculated with 0 2 ml of the virus suspension

In the neutralization tests a constant amount of virus about 100 $TCID_{50}$ and 2 fold serum dilutions were employed Equal parts were mixed incubated at 37° C for one hour and subsequently stored at 4° C until use ordinarily for a period of a few hours only Two roller tubes were used per serum dilution and each tube was inoculated with 0 2 ml of the serum virus mixture

The tubes were incubated at 37° C for 7 days (2 days in a roller drum and subsequently in a stationary position) when the final readings were made Karbers method was used for calculation of titres Virus titres are recorded in log_{10} values and serum titres in log_2 values

Mah/LSc quotient The antigenic difference between the virus strains has been expressed as the difference betwen the mean titre of the 3 Mahoney antisera and the mean titre of the 3 LSc antisera described in the section Antisera (8)

By this calculation the type 1 strains which are closely related to the Mahoney virus become characterized by a positive value while the LSc like strains are characterized by a negative figure

As the standard deviation on the difference between two means each calculated from 3 titres from a single experiment was found to be 0 5 log_2 only differences greater than 1 0 log_2 were regarded as significant ($P < 0.05$)

d and T markers A limited number of strains was examined for d marker (11 13) and T marker (9 10) The *T marker* was studied by virus titrations in roller tube cultures at 34° C 37° C and 40° C using ten fold virus dilutions and 10 cultures per dilution (8) The *d marker* was studied by plaque titration of the strains with 0 11 and 0 40 per cent $NaHCO_3$ in the agar overlay
ployed with two monolayer bottle cultures per dil
with 0 2 ml of the virus dilutions incubated at
with Earle s BSS containing 1 1 per cent agar 0 5
0 1 per cent yeast extract 0 1 per cent bovine albumin 0 0025 per cent neutral red and $NaHCO_3$ in one of the two above mentioned concentrations They were then incubated at 37° C and the plaques were counted the first 5 days the difference in titres being calculated daily

EXPERIMENTS AND RESULTS

All the type 1 polio strains were studied in neutralization tests against the three selected anti Mahoney sera and the three anti LSc,2ab sera

described under materials and methods, and each experiment included as reference strains the Mahoney strain and the laboratory LSc,2ab strain, or the vaccine LSc,2ab strain In Tables 1 and 2 are listed the results of the serodifferentiation tests derived from 16 different experiments

TABLE 1

Intratypic Serodifferentiation Between Various Laboratory Strains of Polio Virus Type 1

Virus strain	No of exp	Mah LSc quotient		
Mahoney	14	+2 9	+2 7	+0 9
		+3 3	+2 0	+3 2
		+2 3	+2 4	+2 1
		+2 0	+2 5	+3 2
		+2 0	+3 4	
Brunhilde	2	+3 2	+2 3	
Chat	3	+1 3	+3 1	+2 5
LSc 2ab	6	−1 6	−2 2	(−1 1)*
		−2 4	−2 0	−1 5
Sabin vaccine Connaught 109 2	5	−1 5	−2 5	−1 8
		−2 8	−2 0	
Sabin vaccine Connaught 110-3	5	−1 7	−4 0	(−1 7)*
		−2 8	−3 0	

* Parenthesis indicates ill defined end points in the serum titrations

Table 1 shows the Mah/LSc quotient for the type 1 laboratory strains studied It will be seen that they fall in 2 distinctly different groups, namely one including Mahoney, Brunhilde and Chat strains, all with a positive Mah/LSc quotient, and the other comprising LSc 2ab and the 2 vaccine lots, 109-2 and 110-3, all with a negative quotient It will also be seen that for each virus strain there is a great deal of variation from one experiment to the other, the figure for Mahoney for instance, varying from 0 9 to 3 4 and for LSc from −1 1 to −2 4 It was, however, found that for each laboratory virus strain the Mah/LSc quotient is either always positive or always negative

In Table 2 the Mah LSc quotient is shown for 49 polio type 1 strains isolated from 35 individuals These are listed chronologically as to the time when the first specimen from the person was received in the laboratory Of these strains, 22 were isolated during 1956-1962 from persons living in different parts of Denmark Sixteen of the strains were isolated from paralytic patients, 2 from cases of aseptic meningitis and 4 from healthy carriers of polio type 1 virus All 22 strains were found to have a positive Mah/LSc quotient

From the spring 1962 to the spring 1963 a limited number of persons was vaccinated with Sabin type 1 oral polio vaccine (LSc,2ab strain), manufactured by the Connaught Laboratories in Canada Faecal specimens were collected at weekly intervals and 18 virus strains from these individuals have been included in the present experiments It can

to 7 years They were typed and titrated and used in their first tissue culture pas sage

Antisera The antisera were produced by inoculation of rabbits with virus without adjuvans 7 ml subcutaneously, 1 ml intradermally and 2 ml intravenously on the same day and bleeding by heart puncture after one week The sera were inactivated at 56° C for 30 min Six selected antisera against type 1 poliovirus strains described in the preceding paper (2) were employed 1) Sera Nos 601 604 and 640 all produced by immunization with the Mahoney strain and in the following called anti Mahoney sera 2) Sera nos 623 610 and 628 produced by immunization with LSc 2ab 10077 II and 10077 VIII, respectively, but in the following simply referred to as anti LSc 2ab sera The two last strains were isolated from a 6 year old healthy boy 4 days and 43 days after he had been fed the Sabin type 1 vaccine They have been found to be serologically closely related to the LSc 2ab strain (2)

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The tubes were incubated at 37° C for 7 days (2 days in a roller drum and subse quently in a stationary position) when the final readings were made Karber's method was used for calculation of titres Virus titres are recorded in log10 values and serum titres in log_2 values

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EXPERIMENTS AND RESULTS

All the type 1 polio strains were studied in neutralization tests against the three selected anti-Mahoney sera and the three anti LSc,2ab sera

be seen that all these strains from vaccinated persons were characterized by a negative Mah/LSc quotient (Table 2) In contrast, 2 strains, Nos 10133 and 10465, isolated from paralytic patients in 1962, both had a positive Mah/LSc quotient

In the last part of Table 2 is listed a total of 9 polio strains isolated from 7 patients vaccinated with oral polio type 1 vaccine during the vaccination campaign in April May 1963 Four of the patients were ho [illegible]

pa [illegible]

LSc quotient and could thus be characterized as belonging to the LSc group It would perhaps seem natural to assume that the 4 cases of meningitis were caused by the type 1 vaccine strain However, no increase in the number of aseptic meningitis cases was observed in the population during or after the vaccination period, 22 April through May 4, when 2 6 Mill out of a total of 4 5 Mill inhabitants were vaccinated It is therefore reasonable to conclude that the presence of vaccine virus in the stools of these 4 meningitis cases is unrelated to the illness of the patients In this connection it should be noted that one patient No 11342 became ill already on the day of vaccination and that the illness of patient No 11427 began 53 days after vaccination

As regards the paralytic patient No 11282, the 3 strains included in Table 2 were isolated 8, 13 and 19 days respectively after the vaccination (4 8 and 14 days after onset of illness) and all 3 isolates showed a positive Mah/LSc quotient They belong therefore antigenetically to the Mahoney group, which is a strong indication that the patient's illness was caused by a Mahoney-related, wild type 1 polio strain and that the Sabin type 1 vaccine, which was administered to the patient 4 days before the onset of illness, had nothing to do with the disease

In order to substantiate this conclusion, strains isolated from the patients were examined as to their T and d markers (9–12) As regards T markers, Table 3 shows the titres obtained for a series of strains cultivated in tissue culture at 34° C, 37° C and 40° C These studies showed that the strains isolated from the paralytic patient 11282 were T+ strains whereas the strains isolated from the other patients were T

Table 4 shows the results of the d marker studies The 2 strains from patient 11282 strain 10133 from a paralytic patient in 1962 and the Mahoney strain were all found to be d+ In contrast, the LSc,2ab vaccine virus and a strain No 10077 isolated from a healthy child vaccinated with oral type 1 vaccine were both found to be d—

Strain 11282 thus clearly differs from the type 1 vaccine strain both in its antigenic pattern and as regards T and d markers, whereas it closely resembles the Mahoney strain and the type 1 virus strains previously isolated from paralytic cases in Denmark

TABLE 2

Intratypic Serodifferentiation between Type 1 Polio Strains Isolated in Denmark 1956 196

Virus strain	Age years	Diagnosis	Stool specimen			Mah I Se quotient
			Date	Day after vaccin	Day of illness	
3273 I	7	paral polio	Sept 56		5	+1 8
II					11	+1 4
3637 II	6	paral polio	Nov 56		16	+2 0
IV					42	+0 5
3648 II	14	paral polio	Nov 56		23	+2 6
3789 I	24	paral polio	Nov 56		8	+0 8
4952 I	31	paral polio	July 58		8	+2 2
4989 II	4	polio contact	Aug 58			+1 0
5253 I	6	meningitis	Oct 58		6	+0 3
5395 I	6	paral polio	Nov 58		8	+3 2
5650 I	8	paral polio	Apr 59		15	+1 7
6652 I	6	meningitis	July 60		2	+2 4
7646 I	5	paral polio	May 61		10	+1 5 +1 2
7794 I	3	polio contact	May 61			+2 2
7798 I	4	polio contact	May 61			+2 5
7803 I	3	polio contact	May 61			+2 2
7920 I	9	paral polio	June 61		12	+2 3
7944 I	6	paral polio	June 61		14	+2 4
III			July 61		28	+2 9
9710 I	14	paral polio	Apr 62		8	+1 8
9892 I	23	healthy	May 62	5		—2 7 —2 1
IV		healthy	June 62	28		—1 8 —0 8
10077 II	6	healthy	Aug 62	4		—2 6 —2 0
VIII		healthy		43		—1 9
10079 II	3	healthy	Aug 62	4		—2 2
10133 I	14	paral polio	Aug 62		7	+2 6 +1 1 +1 7
10465 I	17	paral polio	Oct 62		10	+1 7
11001 III	5/12	healthy	Apr 63	5		—2 1
V		healthy	Apr 63	14		—2 5
11002 III	1	healthy	Apr 63	5		—0 9
V		healthy	Apr 63	14		—2 7
11004 III	2	healthy	Apr 63	5		3 7
V		healthy	Apr 63	14		- 1 8
11038 I	7	healthy	March 63	2		—1 8 —2 9
III		healthy		16		—1 7
V		healthy		44		—3 2
11039 I	5	healthy	March 63	2		—1 8 —1 6
IV		healthy		35		1 5
11040 I	1	healthy	March 63	2		2 5 —2 0
III		healthy		16		—3 0
11261 I	10	meningitis	May 63	11	7	—0 2 1 0
11282 I	13	paral polio	May 63	8	4	+2 2 +2 0 +1 3 +2 1 +1 5 +2 0
III		paral polio			8	+0 5 +3 0
VII					14	+1 7
11285 I	5	meningitis	May 63	10	6	—1 8
11342 I	3	meningitis	May 63	7	7	1 3
11427 I	16	meningitis	June 63	53	4	—0 8 —1 7
N-5067	2	mb cord cong	Apr 63	3		2 2
N-5114	5	enterit chr	May 63	17		—1 3

TABLE 4

I I q e T t es Obt e l o tl H gh I I B carbo te Co ce trat Agar Overlay
l M rkers

V us stra	t g n		Day 1			Day 2			Day 5		I Mark r
		Log I I t per n l a b carbo a e co ce ra o									
		0 4§	0 1	I ff	0 1	0 1	I ff	0 4	0 1	I ff	
11282 I	ja al jt 1963	<3 0	2 4	<0 f	<3 (	2 6	<0 4	<3 0	2 8	<0 2	I+
II*		5 8	5 J	—0 1	6 2	6 4	—0 2	6 6	f 4	0 2	I+
1 133	jaral jt 1962	<4 0	4 5	<—0 5	5 6	5 6	0 0	6 1	6 0	0 1	I+
Mal ney	lab rat ry strain	6 3	6 4	—0 1	6 7	6 6	0 1	7 0	6 J	0 1	I+
I S 2al va cine	C nna gl t I t 110-3	4 5	3 0	1 5	4 7	3 9	0 8	5 2	5 3	—0 1	I—
10077 II	vacci at I I ealthy ch il I	6 5	5 3	1 2	7 0	5 J	1 1	7 2	7 0	0 2	d

Delay I gr vth i I v I I rl at c nc ntrati n
§ I er nt c ce trati n i aga o rlay
I assag aterial fr n c I st I sa nj I

TABLE 3

Temperature Markers

Strain 11282 and Various Other Strains Isolated from Patients Compared with Mahoney and LSc Virus Strains

Virus strain	Diagnosis	Stool specimen day after vaccinat	Virus titre (log10) at temperature 34°	37°	40°	Temperature marker, rct 40
Mahoney Exp 1			8 1	7 7	7 6	T +
2			7 5	7 1	7 3	T +
3			7 5	7 7	7 9	T +
11282 I Stool	paral polio	8 (4)*	4 1	4 5	3 8	T +
I 1 TC pass		8 (4)	6 7	6 9	6 1	T +
III 1 TC pass		13 (8)	7 1	7 3	7 5	T +
VII 1 TC pass		19 (14)	7 3	7 3	5 5	T +
11285 I 1 TC pass	lymf mening	10 (6)	6 7	6 7	2 5	T —
11342 I 1 TC pass	lymf mening	7 (7)	6 7	7 1	1 7	T —
11427 I 1 TC pass	lymf mening	53 (4)	5 9	5 7	1 5	T —
LSc 2ab			8 1	7 7	2 5	T —
Connaught vaccine lot 109 2			7 1	6 9	3 5	T —
Connaught vaccine lot 110 3			7 9	7 7	3 5	T —

* Figures in parenthesis indicate day of illness

SUMMARY

In the present experiments a series of type 1 polio strains has been examined in serodifferentiation tests by a technique described previously (8) This method was found to be technically simple and entirely adequate for distinguishing between "wild" type 1 polio strains isolated in Denmark 1956–1962 and the progeny of Sabin's attenuated type 1, [illegible] which was used for mass vaccination in 1963

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DISCUSSION

In the present studies strains of polio type 1 virus have been tested against 3 selected anti-Mahoney rabbit sera and against 3 corresponding anti-LSc sera The results have been expressed in terms of a Mah/LSc quotient, this being positive for Mahoney-like strains and negative for LSc-like strains (8).

Using these 2 sets of antisera, the studied laboratory strains clearly fell into 2 groups, one including the Mahoney, Brunhilde and Chat strains, the other comprising the LSc,2ab virus as well as the 2 lots of oral vaccine manufactured from this strain

The polio type 1 viruses isolated from patients and healthy virus carriers, including individuals having received oral vaccine could readily be placed in either one or the other group It has previously been found by other investigators (4, 14) that a majority of wild type 1 polio strains antigenetically are Mahoney-like In the present experiments all the polio type 1 strains isolated before the use of live polio virus vaccine was introduced in Denmark were clearly Mahoney-like, i e with a positive Mah/LSc quotient

A limited number of persons was vaccinated with oral LSc type 1 vaccine in 1962–63, before the mass vaccination was started The strains studied from the vaccinees had a negative Mah/LSc quotient, i e they were—as expected—closely related to the vaccine strain LSc,2ab, and no antigenic change towards the Mahoney antigenic character was with the present technique found during the human passage In contrast, 2 strains, Nos 10133 and 10465, isolated from paralytic patients in 1962 had a positive Mah/LSc quotient, as had all the studied wild strains from the previous years

During the vaccination in Denmark of the age groups 6 months-39 years with Sabin type 1 oral vaccine, 2 6 mill individuals were vaccinated, representing approximately 95 per cent of the eligible section of the population

Early in the vaccination campaign, one paralytic polio case occurred This strain (No 11282) was in serodifferentiation tests clearly shown to be unrelated to the LSc,2ab vaccine strain, and studies of d and T markers showed that the virus strain had the characteristics of a wild polio strain It is therefore concluded that the paralytic illness of this patient was caused by a wild type 1 polio strain and had no causal relationship to the vaccination with oral vaccine

It should be noted that with the present technique using only anti-Mahoney sera and anti-LSc sera, the Chat strain—Koprowski's attenuated type 1 polio strain—falls into the Mahoney-Brunhilde group (Table 1) With the use of anti Chat serum, this strain may be distinguished, however, from the Mahoney group (5)

is lifted up and allowed to dry—usually without heating—until the last drop of fluid has dried up. Generally, direct microscope of the growth on the agar has been carried out beforehand.

Leitz Ortholux microscopes and Osram bulbs have been used in all of the experiments. As usual, in examining these often unstained preparations, the intensity of the light and the means of adjusting it must be in order. Sharpest pictures are obtained by tipping the upper lens of the condensor back. The most satisfactory pictures, especially when small bodies are to be examined, have been obtained by using a Leitz objective 70 1 and oculars augmenting 10 ×.

It is practical to see how such an unstained preparation reacts to staining with, for example, a stain such as that used predominantly in these studies, *viz*: methylene blue. We therefore placed the cover slip with the bacteria on to three or four drops of a 0.1 per cent solution of Grubler's methylene blue (manufacturers Chroma Gesellschaft, Stuttgart Untertürkheim) in distilled water on a slide. It can be seen that the poles and sides of the bacilli apparently become stained first and most strongly, finally to become so strongly stained that the difference in colour intensity diminishes. However, the most interesting aspect for us is that during work with young, rapidly growing cultures on "rich"[1] medium, no visible signs of intracellular elements in the cytoplasma are generally demonstrable in few hours' old cultures.

If it is desired to remove the stain or other ingredients around the bacteria, the slide can be rinsed by adding the cleaning fluid to one end of the cover slip and drawing it off at the other end with filter paper of a suitable size. It is important, of course, when working with acid that the microscope table should be protected as much as possible, and therefore the paper squares used for the drawing off process must be cut and placed so that they do not protrude outside the slide. The most practical procedure is to put the first piece of paper along the edge of the cover slip, and the next on top so that a little of the paper protrudes beyond the cover slip. In this way the cover slip is fixed so that a certain field can be observed, and it can be seen what happens to the individual elements (Fig. 1).

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[1] "Rich" medium in this connection means autoclaved broth produced as follows:—
½ kg of minced beef (without fat and tendons),
1 litre of tapwater

th [illegible] r. It is boiled again for 15 minutes with the other half of the water. The two sets of fluid are then mixed and peptone, salt and secondary *sodium phosphate* added.

Boiling and alkalinization with 5 n NaOH are carried out so that the pH after at least 10 minutes boiling (5 minutes before and 5 minutes after the addition of sodium) is about 8.0.

Filtration is made through paper and the fluid is then measured up to the original volume and autoclaved at 125° C for 20 minutes.

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ELEMENTARY METHOD FOR THE DEMONSTRATION OF THE CYTOPLASMIC MEMBRANE IN BACTERIA

By

J Ørskov

Received 17 x 63

Studies in recent years concerning the internal parts of bacteria—particularly the rod-formed—have proved beyond doubt that inside the cell wall another membrane is surrounding the cytoplasma

However, it is generally agreed that it is fairly difficult to demonstrate this cytoplasmic membrane The literature concerning this subject will not be reviewed here, since there would appear to be too much disagreement between the findings of the writer and of others as regards interpretation Thus, discussion of the subject would take up too much space

TECHNIQUE

As in previous studies on morphology, agar microscopy (1) was the basic method in all the examinations Where these concerned the influence of chemicals on the morphology, efforts have also been made to observe directly what happens This has been done by means of the special cover slip chamber method (2) In the earliest studies of this type the special cover slips were "dotted" with paraffin, but in recent experiments Araldit has also often been used These "dotted" cover slips are made as follows Equal parts of the two Araldit components are mixed carefully, for instance at the bottom of a medicine glass The mixture is then dissolved in chloroform to such a density that "dots" of suitable size can be applied to the cover slip by means of a well-pointed paintbrush or a pen with cut off point The cover slips are dried at 37° C and the next day the "dots" are able to tolerate both heating and the majority of fat-dissolving agents (both the slide and the cover slip must be cleaned very carefully before use)

The slides used were about 7 5 × 4 cm and the cover slips 35 × 35 mm with, for instance, 7 × 7 "dots" applied First a block of suitable size is cut out of the agar in a Petri dish containing the bacteria to be examined at the culture stage desired The block of agar is placed on a slide It is practical to allow it to dry for a few minutes, since in that way the bacteria adhere most evenly to the cover slip which now is placed on the block Slight pressure is applied and then the cover slip

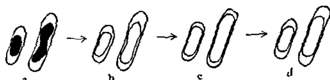


Fig 2

Young bacilli fr m 100 per cent br th agar

a) Treated with 5 n HCl a d
b) Treated with physi l gical saline
c) Treated with NaOH
d) Treated with 0 1 per cent HCl

appearance of the yolk This may, for example sometimes resemble a very thickwalled empty egg shaped bag However, if care is taken to see that growth occurs on a rich medium and examination is made after a few hours growth the picture described above can always be obtained)

If now some drops of weak methylene blue, f inst 0 1 per cent, are added we will see that practically only the yolk is becoming stained

If the unstained yolk preparation is now washed with water containing NaCl it is astonishing to see how small amounts of salt are sufficient to make the yolk swell so that its exterior membrane becomes visible making the ball look empty A solution of f inst 0 001 per cent will suffice NaOH solutions give similar reactions and here 0 0001 per cent is enough Stronger solutions of NaCl and NaOH will make the swellings greater resulting in the yolk membrane reaching the exterior cell membrane and after a longer influence resulting in a contraction of the empty yolk membrane while at the same time the surroundings of of the yolk become uniformly darker, filled with some easily stainable *matter*

If washing is again carried out with weak acid *e g* 0 1 per cent HCl the cytoplasma shrinks again These findings are identical for gram positive (including the angular growing rods) and gram negative bacilli (Fig 2) (As far as the acid fast rods are concerned demonstration is sometimes somewhat more difficult as will be described in a later paper but otherwise there is no difference) Similar reactions are also seen with the protoplasts If for instance Proteus bacilli are cultured on 100 per cent broth agar to which 1 000 units of penicillin per ml have been added easily observable protoplasts are found all over the surface of the agar block already after one hour s growth at 37° C If the air dried cover slip is treated with 5 n HCl and distilled water as described above protoplasts with strongly refractive central yolks appear As in the case of the bacilli these central balls can be made to expand and contract by rinsing with 0 1 per cent NaOH and 0 1 per cent HCl respectively

It might be appropriate to point out the significance of the dotted cover slip technique used here since without the use of that method it

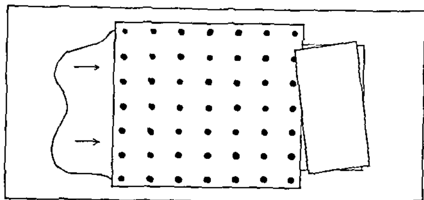


Fig 1
Slide dotted cover slip and arrangement of the first filter papers to fix the cover slip

It is perhaps appropriate to emphasize that much of what has been observed and is to be described in this study are artificial products, but, having observed the occurrence of similar products also in preparations fixed in some of the ordinary ways, it is felt that some new information may have been obtained

As mentioned above, we consider it justificable to state that direct staining of an air-dried, "dotted" cover slip with 0 1 per cent Grubler methylene blue in distilled water, whether it be a young *B subtilis*, which grows well on rich medium, or an *E coli* culture, the result is generally uniform staining of the total bodies (It need only be mentioned that under special growth conditions, and not least with older cultures on rich media, a more motley picture may be seen It can also be stressed that the various stainable elements in such cultures can almost always be seen also in unstained preparations by direct agar microscopy)

Let us now, for example, inoculate a 100 per cent broth agar densely with young culture from identical medium, and after a suitable interval cut out an agar block of suitable size and place it on a slide, let it dry for some minutes, place a dotted cover slip on the surface, press slightly with a fingernail, raise it and let it dry for a couple of minutes, then put it on to three or four drops of 5 n hydrochloric acid applied on a slide, immediately afterwards draw the acid off and wash the preparation with two times ten drops of distilled water When the approximately 20 drops of distilled water have been drawn through the "chamber" by means of squares of filter paper, the preparation can be examined under the microscope It will be seen now that the cytoplasma has apparently contracted to a quite regular spheric or oval, very strongly uniformly refractive "yolk" closely resembling a spore Had this process been observed under the microscope, it would have been seen that no visible changes took place with the cytoplasma before the slide was washed with a d (It should perhaps be added again here that small changes in growth conditions are often of significance as regards the



Fig 4

The picture with the balls arising after treatment with 5 n HCl a d—*Bacillus subtilis* Staining with methylene blue followed by treatment with 5 per cent NaOH

The former seem to contain much more "cell material" than the gram-negative

Another important technical point is the duration of the acid treatment before rinsing with distilled water Briefly, it can be stated that for about 30 seconds no particular change takes place in the described reactions, but after that period an increasing number of seconds causes a gradual decrease of the size of the yolk, presumably as the result of a concentration plus a possible dissolution and removal of some of the original cytoplasmic content If the acid is allowed to work for some minutes before washing with distilled water the cytoplasmic membrane contracts even more, taking the form of smaller balls which can generally be demonstrated to be tiny yolks

Finally, a few remarks concerning some curious observations in connection with decolourization with *e g* 5 per cent NaOH of young rods containing "yolks" stained with methylene blue

The first thing to happen when, for instance, the *B subtilis* yolk is washed with NaOH is that the yolks become bigger and change to a violet bluish colour They then turn reddish to yellowish gradually loosing practically all their colour Subsequently, often after a surprisingly long time, strongly stained small spherical bodies suddenly appear which in form, size and location resemble those found both during growth on poor media and after long exposure to acid, as described above (Fig 4) Apparently the light plays a great rôle in bringing about these stained bodies as they are arising much earlier in the strongly illuminated field than in places more distant from this

Using the same technique similar reactions are found with gram-negative rods For good reasons I shall abstain from trying to explain these phenomena

SUMMARY

When young rods—both gram-positive and gram negative—which grow well on rich medium, *e g* 100 per cent broth agar, are examined by means of the 'dotted' cover slip chamber method, it is found that brief treatment with hydrochloric acid and subsequent rinsing with distilled water causes a strong contraction of the cytoplasma This results in the cytoplasma taking the form of a strongly refractive ball ("yolk") or double ball lying in the centre of the rod Subsequent treatment with saline or alkaline fluid causes an enlargement of the ball and

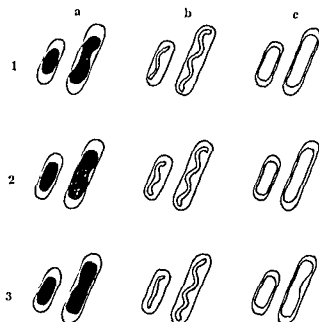


Fig 3

1 *Escherichia coli*, { a) treated with 5 n HCl a d; b) treated with 4 8 n HCl a d; c) treated with 4 6 n HCl a d }
2 *B subtilis* same treatment
3 *Proteus*, same treatment

has not been possible for me to produce the "yolk" phenomenon mentioned above

Heat fixation of dotted Araldit cover slips placed, dots upwards, on a slide with a little drop of water and heated over flame until the cover slip hops off, or f inst fixation with 10 per cent formalin in saline does not much alter the reactions described, while Osmium acid fixation can have a greater influence on the reaction, dependent on the technique used

If at the stage where the cytoplasma has formed itself into a central sphere or into two half-spheres (which means that the central partition is in progress) a few drops of 0 1 per cent methylene blue are added, the spheres become strongly stained and uniformly dark-blue and usually somewhat larger in size The rest of the bacillus is hardly stained at all

It is probably appropriate here to stress that not less than 5 n HCl is necessary to create the described yolk phenomenon The use of for instance 4 8 n HCl gives a quite different picture, while 10 n HCl gives results very similar to those found using 5 n HCl (Fig 3) Some of the reactions caused by concentrations of hydrochloric acid lower than 5 n remind of the picture produced by means of ribonuclease when using the "dotted" cover slip method (3) The very big difference between gram-positive and gram-negative rods can also be seen in this study



Fig 4

The picture with the balls arising after treatment with 5 n HCl a d—*Bacillus subtilis* Staining with methylene blue followed by treatment with 5 per cent NaOH

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When young rods—both gram positive and gram negative—which grow well on rich medium, *e g* 100 per cent broth agar, are examined by means of the "dotted" cover slip chamber method, it is found that brief treatment with hydrochloric acid and subsequent rinsing with

the appearance of a clear surrounding membrane Exposure to weak hydrochloric acid solution causes a certain contraction

Staining of the bacilli with such central "yolks" results in only the latter becoming stained Decolourization by means of suitable concentrations of alkaline fluid causes the appearance of quite small spheric structures located at the centre of the "empty" yolk-sack

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THE ACID FASTNESS OF BACILLI AND THE CYTOPLASMIC MEMBRANE

By

J ØRSKOV

Received 17 x 63

A previous investigation in collaboration with *Jensen* in 1926 showed that the acid fastness of a certain bacillus was absolutely dependent on the medium used, for example, transfer from Besredka medium, where almost all the elements were acid fast to ordinary broth agar caused *all the acid fastness to disappear*

We considered it justifiable to conclude that acid-fastness was connected with membrane permeability (1)

The following brief description of experiments with a great number of acid fast rods, both pathogenic and non-pathogenic, would seem to show that the above mentioned conclusion is not *completely improbable* Let me stress that perhaps there is nothing original about these findings, but as knowledge of them seems to have become somewhat obscure nowadays, it would probably be advantageous to freshen it up

Before discussing the question of acid fastness, I should like to mention a large group of acid fast bacilli, which, in the same way as the tubercle bacilli, belong to the angular growing, often branching microorganisms, but which are characteristic in that morphologically they are often very pronouncedly "granulated" This is so evident in some cases that both by direct agar microscopy and in Ziehl Neelsen slides one might be tempted to liken the inferior of the bacilli to a string of beads A similar picture can also be encountered—though it is not nearly so obvious—in examination of, for instance, BCG, human, bovine and avian strains of tubercle bacilli

The majority of the strongly *granulated, acid fast bacilli in question* were sent to *Statens Seruminstitut via WHO from* African patients with lung lesions judged to have been caused by these microorganisms

Out of these approximately 30 strains one has been chosen which shows this bead picture particularly clearly (No 223), direct agar microscopy revealing all stages *from intracellular* strings of beads, via short cylindrical elements, to rods which are uniformly refractive from one pole to the other Ziehl Neelsen staining gives absolutely identical findings and only the beads—the cylinders—and the homogeneous rods are acid fast

the appearance of a clear surrounding membrane Exposure to weak hydrochloric acid solution causes a certain contraction

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become acid fast again Repeated washing with chloroform gives the same result as mentioned above and generally it is necessary to repeat the process three to four times before the bacilli remain non acid fast after *Ziehl Neelsen staining*

A similar result can be obtained, for example, with ether or certain phenol concentrations

When the bacilli have become definitely non acid fast, they can be made acid fast again by the addition of various kinds of fat

The first of these *experiments* was carried out with chloroform extract of BCG culture from Dubos agar by the simple method of wrapping the dry bacilli up in a little wedge of filter paper and lowering it into a test tube containing chloroform After a short extraction time, it was sufficient to add a drop of the chloroform to the dried non acid *fast slide and let the drop dry leaving* a slightly misty film over the bacilli When Ziehl Neelsen staining is repeated, the previous acid-fastness is recovered If we take, for instance, strain No 260 in its non-acid fast phase, we can similarly cause these bacilli to become acid fast by means of a little chloroform extract originating from the BCG culture *Exactly the same result as described here* can be obtained with all the acid fast bacilli examined including BCG, human, bovine and avian tubercle bacilli

In my opinion these findings are in support of the assumption that differences in membrane permeability must be the most important factor in the acid fastness and that the *internal* cytoplasmic membrane plays the most imported role It would also seem to be clear that fat is of significance for this permeability—as indeed has always been supposed'

It may be added that any definite question of specificity as regards the lipoids to be chosen in order to obtain acid fastness is apparently not involved Human sebaceous gland fat, margarine and butter, are able to reproduce the acid fastness of all of the widely varying acid fast *rods* examined (A single test on a minoscale using a Subtilis strain to see whether it was possible to transfer acid fastness to a bacillus not belonging to the acid fast variety gave an absolutely negative result)

SUMMARY

The cytoplasmic membrane (2) which can be demonstrated in the non acid fast bacilli seems to be able to make itself manifest in certain, often strongly granulated rods in which the internal bodies are visible by means of direct agar microscopy and Ziehl Neelsen staining These internal bodies can be strongly assumed (1), *the acid fastness* seems to be connected with the membrane, fat being the important factor which influences the permeability of these membranes

All *acid fast* rods can be made non acid fast by means of various fat dissolving substances *e g* chloroform



Fig 1.

No 223 a) *Direct agar microscopy (and Ziehl-Neelsen)*
b) *After treatment with some drops of distilled water*

If now the dotted coverslip is lifted, airdried and placed on some drops of distilled water, the b-picture is obtained in which all the intracellular elements have augmented in size, sometimes nearly reaching the outer cellwall The morphological parallelism with the double-membrane findings in non-acid-fast rods seems striking

As mentioned, I have found identical conditions with pathogenic, acid-fast rods, though not nearly so pronounced or as frequent

As to the question about the factors causing the acid-fastness, we can take as starting point strain No 260, called Smegma bacillus, which after 3–4 days' growth on Dubos agar at 37° C becomes uniformly acid-fast, as shown by the Ziehl-Neelsen technique However, if the growth of the same strain is observed at room temperature on the same medium, it will generally be found that it is not acid-fast during the first days At that stage it is easily stainable without heating by means of both carbolfuchsin and, for example, 0 1 per cent methylene blue However, this colour fixation disappears immediately, even as the results of exposure to weak acid, as can be oberved directly under the "dotted" coverslip

On the other hand, the acid-fast form is the same as that of the other acid-fast rods, in that only few are stainable without heating, again suggesting differences in membrane permeability (Here it should be interposed that it can be seen with certain relatively marked acid-fast rods that, in the presence of acid, *e g* after carbolfuchsin staining, the colour changes from red to yellowish—as also happens with the stain outside the cell during acidification—and then changes back to deep red after washing with distilled water I do not know what happens biochemically here In the case of other, often more acid-fast rods, this change i colour does not take place as the result of the action of acid)

If we return to strain No 260 in its relatively acid-fast phase and make a Ziehl-Neelsen stain, we can change its acid fastness, *e g* by means of chloroform Continued washing with chloroform does not change the deep red colour to any extent, but the acid fastness disappears At the same time it is easy to demonstrate under the "Araldite dotted" coverslip that after washing with distilled water the bacteria on the slide can be stained without heating and the colour can easily be removed again by means of even weak acid If new Ziehl-Neelsen staining is carried out with this non-acid-fast preparation, all the bacilli

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STUDIES ON TRANSFORMATION IN MORAXELLA AND ORGANISMS ASSUMED TO BE RELATED TO MORAXELLA

I A *Method for Quantitative Transformation in* Moraxella *and* Neisseria, *with Streptomycin Resistance as the Genetic Marker*

By

Kjell Bøvre

Received 7 xii 63

As expressed previously (*Catlin & Cunningham* 1961, *Bøvre & Henriksen* 1962, *Bøvre* 1963), there are reasons to believe that transformation studies in *Moraxella* and *Neisseria* may be applied to the clarification of the relationships of these bacteria, in addition to the obvious opportunities of using members of these groups in the study of basic genetic principles

It is planned to develop a reliable quantitative method of transformation for the application in the following studies, which will appear in succession Ratios of transformation frequencies will be determined among a relatively great number of *Moraxella nonliquefaciens* strains, classified according to conventional criteria, to see whether there is homogeneity in terms of transformation In the case of heterogeneity, efforts will be made to correlate the transformation results with other possible criteria of differentiation between strains of dissimilar genetic affinities Studies of the same kind will be performed in other bacteria with possible relation to *Moraxella nonliquefaciens*, and transformation ratios will be determined between genetically homogenous groups, if possible Accordingly, classification of these bacteria will be discussed

The primary aim of the present paper is to analyse and eventually correct a previous tentative method (*Bøvre & Henriksen* 1962) in order to prevent variation of results as a consequence of methodological instability Less attention is for the present paid to exactly optimal conditions, when the latter are not considered necessary to obtain comparable results As there are individual features which might come into consideration when new recipients and donors are included, it may be necessary later to perform supplementary methodological investigations

Whether dependent on growth or produced artificially, non acid fastness can be recovered by means of the addition of chloroform extract from, for instance, BCG culture However, the lipoid substance required is not particularly specific in nature, since many other lipoids *e g* of human origin and margarine or butter, can be used for restoration of lost acid fastness

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FURTHER DESCRIPTION OF THE METHOD AND STUDIES ON SOME OF ITS ASPECTS

DNA Preparation

Each selected mutant decided to be used as donor in transformation experiments, is subjected to the following procedure The surface growth of 15 blood agar Petri dishes is harvested after incubation at 32 33° C in a humid atmosphere for 20 h (if the strain in question grows more slowly than the average number of plates and incubation time are adjusted correspondingly) The bacteria are suspended in 32 ml of citrate buffered saline (0 14 M NaCl + 0 015 M sodium citrate, pH 7 4) This is the standard buffer described by *Zamenhof, Alexander & Leidy* (1953) The wet weight of the suspended bacteria is approximately 0 8 g 0 3 g of sodium dodecyl sulphate is added and the suspension placed in a 56° C water bath for 10 min, followed by cooling at room temperature for 30 min After cooling, 8 ml of 5 M sodium perchlorate and 40 ml of chloroform with 4 per cent iso amyl alcohol, are added Then follows mechanical shaking at 5° C for 30 min The clear or almost clear supernatant resulting from subsequent centrifugation at 5000 RPM in a swinging bucket centrifuge for 30 min at room temperature is shaken once more with an equal volume of chloroform iso amyl alcohol for 15 min at 5° C and centrifuged as before As a rule only little protein is now seen at the interphase The last supernatant is precipitated with 2 volumes of 96 per cent ethyl alcohol and the floating and coherent precipitate is collected by winding it up with a closed Pasteur pipette The excess of alcohol is drained off by pressure and rotation against the vessel wall, followed by soaking in 75 per cent ethyl alcohol and a subsequent drainage in the same manner The precipitate is then loosened from the pipette into 5 10 ml of standard buffer where solution is allowed to take place at 5° C till the next day The remaining lumps are dispersed by gentle pipetting and the solution, which is clear or almost so, is eventually further diluted to give a slightly viscous appearance, falling drops followed by a thin thread of the solution This concentration corresponds to a total DNA content of approximately 200 µg per ml, as measured with the conventional diphenylamine reaction of Dische The solution is stored at −20° C or at 5° C till use When during transformation experiments the DNA solution has been diluted tenfold saturating levels of active transforming principle have always been present at various recipient densities during 15 min of exposure

It may be asked whether an apparently stable procedure will secure genetically representative results in comparative transformation. Eventual deviation in terms of transformation by single strains or groups of strains might perhaps be due to factors independent of general composition of the genome itself. These possibilities will be partly discussed in the present communication, but will be more completely investigated in connection with results obtained through a wide application of the method.

The outlines of the method. Mutants resistant to at least 500 μg of streptomycin per ml are selected, and active extracts of transforming DNA are prepared. Streptomycin sensitive recipient bacteria with sufficient, not necessarily optimal competence of being transformed are then subjected to the action of transforming DNA in fluid medium for a fixed period of time. The action of DNA is stopped with deoxyribonuclease, and appropriate dilutions of the mixture are spread on the surface of solid medium in Petri dishes. After incubation till full phenotypic expression of streptomycin resistance in the cells transformed, the intact medium is moved by means of a metal spatula and a metal spade into another Petri dish of the same size, containing beforehand a layer of agar with sufficient streptomycin to give 500 μg per ml after diffusion has taken place. Incubation is continued for 3 days and colonies arising from transformed cells are counted and controlled. In all experiments where ratios of transformation frequencies are to be determined, aliquots of the same recipient population are exposed simultaneously to the different DNA extracts. The effectivity of deoxyribonuclease treatment is controlled by means of parallel tubes with DNA which has been treated with deoxyribonuclease for 10 min before the addition of the recipient. A more complete description of the method to be analysed has been given in the previous communication by *Bøvre & Henriksen* (1962).

MATERIAL

The results to be presented here are partly based on application of the method in a great number of strains of *Moraxella nonliquefaciens*, *Moraxella bovis* and *Neisseria catarrhalis* (*Bøvre*). This has given a background of experience which has been of importance when evaluating the applicability of the method. 4 strains supposed to be representative were chosen as main subjects in the study of single factors of the procedure. These were *Moraxella nonliquefaciens* 4663/62 (a strain isolated from the nasal cavity of an inpatient at Rikshospitalet, Oslo), *Moraxella nonliquefaciens* 7784 (a strain received from the National Collection of Type Cultures, London), *Moraxella bovis* 10900 (an American Type Culture Collection strain) and *Neisseria catarrhalis* Ne 11 (a strain received from dr. B. W. Catlin). All 4 strains are named according to conventional biochemical, cultural and morphological criteria. It must be mentioned here that the strain *Moraxella nonliquefaciens* 7784 is named *Moraxella lacunata* in the National Collection of Type Cultures. However, examination by the author has revealed the strain to be a somewhat slow growing *Moraxella nonliquefaciens*.

The streptomycin resistant mutants used in this study were selected in the following manner. The sensitive strain in question was heavily seeded on blood agar. Following incubation till growth was clearly visible, the medium was layered on

ntration of 500 µg of streptomycin per
n place Incubation was continued and
resistance The mutants obtained were
at least 1000 µg of streptomycin per ml

This method of mutant selection is analogous to the selection of transformants and is the only procedure applied at present

FURTHER DESCRIPTION OF THE METHOD AND STUDIES ON SOME OF ITS ASPECTS

DNA Preparation

Each selected mutant decided to be used as donor in transformation experiments is subjected to the following procedure The surface growth of 15 blood agar Petri dishes is harvested after incubation at 32 33° C in a humid atmosphere for 20 h (if the strain in question grows more slowly than the average, number of plates and incubation time are adjusted correspondingly) The bacteria are suspended in 32 ml of citrate buffered saline (0 14 M NaCl + 0 015 M sodium citrate, pH 7 4) This is the standard buffer described by *Zamenhof, Alexander & Leidy* (1953) The wet weight of the suspended bacteria is approximately 0 8 g 0 3 g of sodium dodecyl sulphate is added and the suspension placed in a 56° C water bath for 10 min, followed by cooling at room temperature for 30 min After cooling, 8 ml of 5 M sodium perchlorate and 40 ml of chloroform with 4 per cent iso-amyl alcohol, are added Then follows mechanical shaking at 5° C for 30 min The clear or almost clear supernatant resulting from subsequent centrifugation at 5000 RPM in a swinging bucket centrifuge for 30 min at room temperature, is shaken once more with an equal volume of chloroform iso amyl alcohol for 15 min at 5° C and centrifuged as before As a rule only little protein is now seen at the interphase The last supernatant is precipitated with 2 volumes of 96 per cent ethyl alcohol and the floating and coherent precipitate is collected by winding it up with a closed Pasteur pipette The excess of alcohol is drained off by pressure and rotation against the vessel wall followed by soaking in 75 per cent ethyl alcohol and a subsequent drainage in the same manner The precipitate is then loosened from the pipette into 5 10 ml of standard buffer where solution is allowed to take place at 5° C till the next day The remaining lumps are dispersed by gentle pipetting and the solution which is clear or almost so is eventually further diluted to give a slightly viscous appearance falling drops followed by a thin thread of the solution This concentration corresponds to a total DNA content of approximately 200 µg per ml, as measured with the conventional diphenylamine reaction of Dische The solution is stored at —20° C or at 5° C till use When during transformation experiments the DNA solution has been diluted tenfold saturating levels of active transforming principle have always been present, at various recipient densities during 15 min of exposure

The previous method of DNA extraction (*Bøvre & Henriksen* 1962) was mainly in accordance with the first steps of the procedure applied by *Catlin* (1960) The use of sodium dodecyl sulphate as lysing and partially deproteinizing agent has been maintained, but the main lines followed are now those of the method described by *Marmur* (1961) The method regularly gives a good yield of DNA, easily precipitated and floating coherently in alcohol

It is considered of great importance to avoid denaturation of DNA during extraction, and therefore as gentle a procedure as possible has been applied The temperature of 56° C for 10 min which is used to facilitate lysis, is well below temperatures found to influence the physical, chemical and biological properties of native DNA (*Zamenhof, Alexander & Leidy* 1953, *Lerman & Tolmach* 1959, *Marmur & Doty* 1959, *Eigner, Boedtker & Michaels* 1961 a o)

Experiments were performed to show whether transformation frequencies at DNA saturation were influenced by different degrees of purification of DNA, both in heterologous and homologous systems In one experiment an opaque solution of DNA, deproteinized only once, was compared with an extract deproteinized 4 times, in heterologous transformation of *Moraxella nonliquefaciens* by a *Moraxella bovis* donor The effect of these two extracts did not differ in the yield of transformants, regardless of the DNA exposure time In another experiment, a *Moraxella bovis* DNA extract, deproteinized 2 times, was compared with a corresponding extract subjected to 5 deproteinizations, including RNase treatment No significant difference was observed between these two extracts as concerns their transforming activities at saturation in homologous reactions The results of these experiments are presented in Fig 1 It is concluded that there are wide limits concerning the degree of purification when the reliability of determined heterologous and homologous transformation frequencies are considered at saturation with DNA at least when the strain studied here is used as donor

Spizizen (1958), working with *Bacillus subtilis*, found that deproteinization reduces the minimal amount of total DNA required for transformation to occur, probably due to close association between DNA and protein which causes a large molecular complex On the other hand, he also found that RNase treatment during purification of DNA had a reducing effect on number of transformants per weight unit of total DNA He did not try to demonstrate the transforming activity at saturation of different extracts of the same strain *Lerman & Tolmach* (1957) found a slightly higher transformation frequency in pneumococci when using crude DNA extracts, as compared with purified DNA

Schaeffer (1958) pointed out that it is conceivable that the same extraction procedure might lead to fully active preparations when applied to one donor strain, and to partly inactivated ones when applied to other strains *Hotchkiss* (1957) presented evidence that the saturation yield

TRANSFORMANTS PER PLATE: 20, 40, 60, 80, 100, 120

DNA EXPOSURE N MINUTES: 5, 10, 15, 20, 25, 30, 35, 40

Fig 1

The effect of deproteinization of extracts Lower set of curves The effect at saturation of *Moraxella bovis* 10900 DNA extracts in the transformation of *Moraxella nonliquefaciens* 7184 Continuous line 1 depr [illegible] led line 4 depr [illegible] *axella bovis* 10900 deproteinizations [illegible] atment

of transformants is a measure of the quality of a DNA preparation, *i e* its ability to supply active DNA bearing a certain marker relative to its total DNA content *Ephrussi Taylor* (1957) pointed to the fact that along with molecules bearing the selective marker in a DNA solution there is an unknown number of molecules which lack the selective marker In addition to this *inherent heterogeneity there are inactivated* molecules and in some instances fragments of molecules which will exert their interfering effect in transformation experiments

According to these considerations it is theoretically possible that DNA extracted from different strains or groups of strains are characterized by different proportions of transforming marker DNA units to non marker DNA units The results presented in this section indicate that such differences eventually would depend on other factors than fluctuations of the degree of denaturation during the extraction procedure described

The eventual characterization of individual DNA extracts by different contents of competitive non marker DNA would of course complicate quantitation However, a discussion of that question is superfluous in a case where it can be shown that the transformation ratio is the same when using the heterologous donor as recipient in an analogous experiment This confirmation will be provided if possible whenever the significance of transformation ratios are considered in the future

To test the eventual toxicity of DNA extracts on the recipient popula

tions, a strain of *Moraxella bovis* was exposed to 5 different DNA extracts of *Moraxella bovis, Moraxella nonliquefaciens* and *Moraxella lwoffi* for 60 min under conditions simulating the transformation procedure, except for the prolonged exposure No reduction in total counts was observed as compared with controls not treated with DNA From this is concluded that DNA or remnants of reagents from the extraction procedure described most probably exert no deleterious effect on the recipient population during transformation

Recipient Cells

A young blood agar surface culture (4–7 h incubation at 32–33° C in a humid atmosphere) is harvested with a loop and emulsified in broth (see below) by vigorous repeated expulsion from a Pasteur pipette to make the most homogenous suspension possible The suspension is immediately placed in divided portions at —20° C and stored at this temperature till use

Accurate studies on variation of competence related to growth conditions before storage have not yet been performed However, the general impression is that young blood agar cultures are more competent recipients than older ones A transformability of the order 0 1–5 per cent of recipient cells has regularly been obtained in several strains

Preservation of transformable bacteria at low temperatures is known from transformation of pneumococci (*Fox & Hotchkiss* 1957) These authors used glycerol preserved recipients stored at —20° C Initial experiments with *Moraxella nonliquefaciens* 7784 suggested that glycerol at least was not beneficial for storage, and therefore storage has been performed without the addition of glycerol Under these conditions there is only a slight reduction in total count and only a proportionate reduction in competent cells during storage for 7 days For practical purposes this is a sufficient period for performing several sets of quantitative transformations with the same recipient

The Media for Storage of Recipients, Transformation, Phenotypic Expression and Selection of Transformants

Both for storage of recipients and for transformation is applied Brain Heart Infusion Broth (Difco), to which are added 0 0005 M $CaCl_2$ and 0 2 per cent bovine albumin (fraction V) Neither Ca^{++} nor bovine albumin is indispensable for transformation to occur in *Moraxella* and *Neisseria catarrhalis* However, the general impression has been that these supplements augment the transformant yield and make it easier to predict the number of transformants

Moraxella bovis 10900 was subjected to the following experiment Samples with equal inocula in each of 4 media, Brain Heart Infusion Broth and the same medium supplied with 0 0005 M $CaCl_2$, 0 2 per cent bovine albumin or (0 0005 M $CaCl_2$ + 0 2 per cent bovine albumin)

respectively, were counted for viable cells after storage for different periods of time. All counts were reduced from $5.0 \cdot 10^6$ on the second day to $1.2 \cdot 10^6$ on the 7th day. Thus, Ca^{++} or albumin do not seem to be of importance for the maintenance of viability at $-20°$ C.

Ca^{++} improved growth of the four test strains considerably when added to the medium in the *above mentioned concentration*. Albumin exerted an additional growth promoting effect in some instances. In the complete medium the minimal inoculum per ml required for the initiation of growth of *Moraxella nonliquefaciens* 4663/62 was slightly less than the minimal inoculum giving rise to colonies on blood agar surface in a humid atmosphere. This rough orientation concerning the quality of the medium is considered sufficient for its use in experiments without bacterial multiplication.

In pneumococcal transformation, both Ca^{++} and albumin have been found important for the development of competence and for the fixation of DNA (*Fox & Hotchkiss* 1957). In streptococcal transformation, albumin or serum have been found indispensable for the development of a competence provoking factor (*Pakula & Walczak* 1963). In *Haemophilus*, *Goodgal & Herriott* (1961) in some instances found a stimulation of transformation by bivalent cations as Ca^{++} and Mg^{++}. They also found a slight restoring effect of albumin on the competence of washed bacteria. On the other hand, albumin was found to be inhibitory in the transformation of *Bacillus subtilis* (*Spizizen* 1958), whereas Ca^{++} and other bivalent cations were stimulatory (*Young & Spizizen* 1963).

For the combined phenotypic expression and scoring of transformants is used blood agar, consisting of Tryptose Blood Agar Base (Difco) and 5 per cent citrated human blood, adjusted to pH 7.6, in a volume of 20 ml to each Petri dish. The layer of streptomycin agar, upon which is placed the culture medium after phenotypic expression has taken place consists of Brain Heart Infusion Broth (Difco) with 1.4 per cent agar and 3 mg streptomycin per ml in a volume of 4 ml per Petri dish.

Diffusion and Effective Concentration of Streptomycin

100 plane bottom Petri dishes of the type used in assays, selected at random had diameters ranging from 90 to 91 mm. These values express a uniformity in the area of plates sufficient to secure that the variation in the thickness of given volumes of medium is insignificant when considering diffusion time.

To test the diffusion of selective agent and the actual selection concentration, the following experiments were performed. 10 non inoculated and randomly selected Petri dishes were placed in the incubator at 32–33° C immediately after the initiation of diffusion through lifting the blood agar layer on to the streptomycin agar layer. At appropriate intervals circular filter paper disks, 4.5 mm in diameter, were moistened by contact with the surface of the blood agar at different locations of the various plates. The antibiotic concentration in the filter paper disks

was tested immediately thereafter on surface cultures of the 4 wild type test strains The latter had been preincubated at 32–33° C for 6½ h After the application of the moistened filter paper disks, incubation was continued at 32–33° C for 4 days. It was shown that in 30 min all tested locations of the 10 assay plates revealed diffusion to such a degree that the moistened disks subsequently gave a distinct zone of complete inhibition of further growth of the streptomycin sensitive test strains. Within 60 min. the assay plates revealed maximal diffusion, the moistened filter paper disks invariably giving the same zone diameters as control disks moistened on the surface of blood agar medium prepared with 500 µg streptomycin per ml There was no variation in the surface concentration of streptomycin during 4 days of incubation at 32–33° C, even in the presence of heavy surface growth at the time when diffusion was initiated

Conclusively, it can be stated that the growth of untransformed bacteria is stopped within 30 min at this temperature, even when in confluent visible growth at the time when diffusion of streptomycin begins The calculated final concentration of streptomycin is reached in 60 min from the start of diffusion and this concentration is stable during the maximal incubation period of the transformation procedure

The Lifting of the Blood Agar Layer

When phenotypic expression is finished, a 1 mm broad peripheral rim, free of bacteria, is sliced off by means of a small and sharp metal spatula (this is done to facilitate adjustment to the new dish) Then the spatula is used to raise part of the periphery of the blood agar, and a larger, oval metal spade is gently moved underneath the blood agar till its edge reaches somewhat beyond the center of the dish, while the friction between the agar and the spade is lessened by means of the spatula The agar layer is lifted and lowered on to the streptomycin agar layer, where it is left after having been removed from the large spade by means of the spatula Thereafter, the spatula is gently pressed around the periphery to accomplish full contact between the two agar layers Lack of contact can easily be observed from the under side, and if it should occur near the center, an air bubble can be removed by perforating the blood agar layer with the spatula The tools are sterilized by flaming with alcohol Contamination during handling as a rule does not occur

When relatively fresh blood agar is used, this method is complicated by the extrusion of fluid on the surface during handling When blood agar which has been stored in the refrigerator for 3 6 days, is used, the surface is moistened lightly for only a second This occurrence of surface moisture has never entailed spread of colonies On the other hand, the unexperienced worker may press the agar and pull it during handling, so that rows of colonies arising from single clones may occur

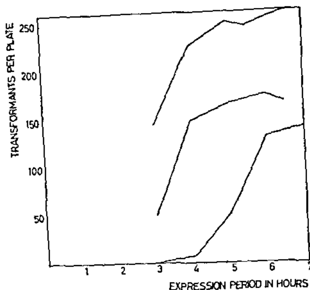


Fig 2

With cautious manipulations, however, any spread of transformant colonies can be avoided This has been controlled in a number of experiments with low counts, where groups of colonies have been absent

Phenotypic Expression

This is allowed to take place at 32–33° C in a humid atmosphere (20 cm² of free water surface per liter of incubator volume).

The four test strains were transformed homologously with DNase treatment at the end of transformation One heterologous transformation was also undertaken, the transformation of *Moraxella nonliquefaciens* 7784 with DNA extracted from *Moraxella bovis* 10900 The latter transformation was simultaneous with the homologous transformation of the same *Moraxella nonliquefaciens* strain, so that expression could be compared in homologous and heterologous transformations under identical conditions

The results are shown in Figs 2 and 3 In these experiments the storage of recipients had lasted only a couple of days, so that the periods of time required to reach a certain stage of growth probably represent minimal values under the conditions of the procedure It was found that expression was not finished until an even distribution of microcolonies was clearly visible to the naked eye. The practical consequence has therefore been that the beginning of streptomycin diffusion is withheld in all experiments till this stage of growth has been reached, *i e*

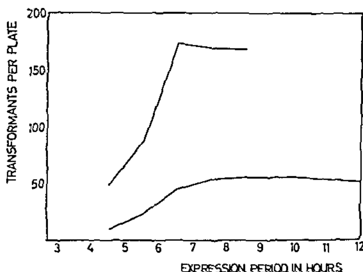


Fig 3

Time required for phenotyp heterologous transformation *nonliquefaciens* 7784 Lower *nonliquefacien*

5–9 h from the start of growth Although a considerable postponement of streptomycin addition did *not bring about a reduction* of scored transformants in these experiments, attention must be paid to the possibility that transformants are partly exhausted due to competition with untransformed bacteria (*Schaeffer* 1956) Therefore, an unnecessary prolongation of the expression period must be avoided

It was observed that the period of time for phenotypic expression in heterologous transformation is identical with that of homologous transformation Therefore, wrong conclusions concerning ratios of heterologous to homologus transformation most probably cannot be accounted for by different periods of time required for phenotypic expression in these bacteria This is in accordance with findings in *Haemophilus* (*Schaeffer* 1956) However, the principal question whether the lower frequency of heterologous transformation as compared with homologous transformation, depends on lessened viability of heterologous transformants (*Schaeffer* 1958), is intimately connected with growth competition between transformants and untransformed bacteria during the expression period As the inoculum is greater in heterologous transformant scoring, the cultural conditions during phenotypic expression may be a most critical step for the correct measurement of heterologous to homologous transformation ratios

The State of the Recipient Population Following Storage

The exposure to DNA is performed in a water bath at 32 33° C, identical with the subsequent incubation temperature This temperature has been chosen because it is near the optimal growth temperature for

many of these bacteria (*Audureau* 1940, *Henriksen* 1952, *Murray & Truant* 1954) and because temperatures in this region have been found to be optimal or not far from optimal in transformation of other bacteria (*Fox & Hotchkiss* 1957, *Lerman & Tolmach* 1957, *Goodgal & Herriott* 1961, *Young & Spizizen* 1963) To secure that this temperature is not critical for transformation in the bacteria under study, the transformation of *Moraxella nonliquefaciens* 7784 by DNA extracted from *Moraxella bovis* 10900 was compared at 29° C, 32° C and 37° C respectively, all other factors equal No significant difference in transformant yield was observed, and the temperature 32–33° C has been maintained

Following removal of the recipient population from —20° C it takes approximately 15 min in the 32 33° C water bath to reach this temperature On no occasion, during repeated experiments, growth of the four recipient strains has been observed during the first 60 min after 32 33° C has been obtained This fact is based on turbidity measurements and on counts of viable cells After that time growth has been observed in some instances as early as 90 min after the initial 15 min

The stability of competence during the period actual for the performance of transformation experiments, which is one of the prerequisites for a quantitative procedure (*Hotchkiss* 1957), was examined in all the test strains in the following manner Beginning at the time when 32 33° C had been obtained (15 min after removal from —20° C), each population was transformed homologously with saturating concentrations of DNA for 5 min intervals at different points of time during the stay at 32–33° C The DNA exposure was each time stopped with DNase and transformants scored after complete expression of phenotype The results are collected in Fig 4 It can be seen that transformability of all recipients keeps remarkably constant from 20 to 65 min after the initial 15 min temperature adaption, and the first part of this stable plateau has subsequently been preferred for the initiation of transformation

The Exposure to DNA and Its Duration

At the appropriate time 0 5 ml of the recipient is added to 0 5 ml of a mixture of transformation medium and DNA, as a rule 0 4 ml transformation medium and 0 1 ml DNA solution To secure saturation, 2 or 3 concentrations of DNA are applied in parallel After the fixed period of DNA exposure, the effect of the latter is stopped by means of 0 05 ml of DNase solution, containing 50 µg DNase The mixture is kept at 32 33° C for 5 min more and then placed in an ice water bath and immediately plated on the surface of blood agar medium, in appropriate dilution which is undertaken in transformation medium, in order not to complicate the system with differences in medium factors between those populations that need dilution and those that *do not* As men-

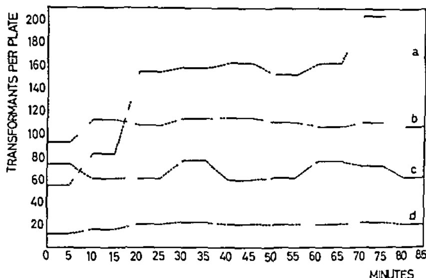


Fig 4

Transformability at 32–33° C following storage at —20° C 0 at the abscissa indicates 15 min after removal from —20° C —— 5 min DNA exposure periods   Intervals not examined a *Moraxella nonliquefaciens* 4663/62 4 days storage b *Moraxella nonliquefaciens* 7784, 7 days storage c *Neisseria catarrhalis* Ne 11 5 days storage d *Moraxella bovis* 10900, 2 days storage

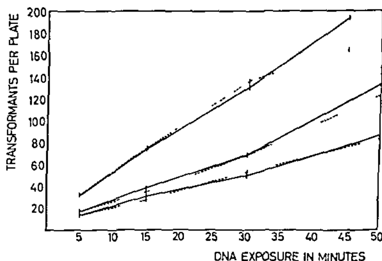


Fig 5

Variation of transformant yield with the duration of DNA exposure The transformations are started at 40 min after removal of recipients from —20° C Upper set of curves Homologous transformation of *Neisseria catarrhalis* Ne 11 Middle set of curves Homologous transformation of *Moraxella nonliquefaciens* 4663 62 Lower set of curves Heterologous transformation of *Moraxella nonliquefaciens* 4663 62 with *Moraxella bovis* 10900 DNA The middle and lower set of curves represent simultaneous experiments Stippled lines Lowest DNA concentrations for saturation control (20 µg per ml in the upper experiment 10 µg per ml in the two others) Continuous lines Highest total DNA concentration (approximately 40 µg per ml)

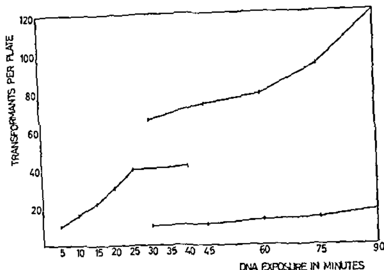


Fig 6

Variation of transformant yield with the duration of DNA exposure The transformations are started 35 min after removal of recipients from —20° C. Middle curve Heterologous transformation of *Moraxella nonliquefaciens* 7784 with *Moraxella bovis* 10900 DNA Upper curve Homologous transformation of *Moraxella nonliquefaciens* 7784 Lower curve Heterologous transformation of *Moraxella nonliquefaciens* 7784 with *Moraxella bovis* 10900 DNA The upper and lower curves represent simultaneous experiments

tioned before, transformation ratios are only determined when using aliquots of the same recipient population simultaneously for all donors Dilution is always the same during transformation for all donors, regardless of their individual expected potency in the transformation of the recipient in question This procedure secures that comparisons are not made between DNA effects on different numbers of recipient cells in the respective parallels

Questions of importance are how the number of transformants behaves as a function of the duration of DNA exposure in this system and whether ratios of transformation frequencies may vary with the duration of DNA exposure

In Fig 5 is presented an experiment of homologous transformation in *Neisseria catarrhalis* Ne 11 at two DNA concentrations A linear increase of transformants is observed during the observation period (45 min) The lowest DNA concentration, which is saturating during 30 min exposure in this experiment and also has been saturating in other experiments of short duration, gives a slightly lower yield of transformants when exposure is prolonged beyond 30 min

Fig 5 also shows the results of another experiment, where *Moraxella nonliquefaciens* 4663 62 was transformed homologously and simultaneously heterologously by *Moraxella bovis* 10900 at saturation with DNA It can be seen that the yield of transformants rises linearly with time

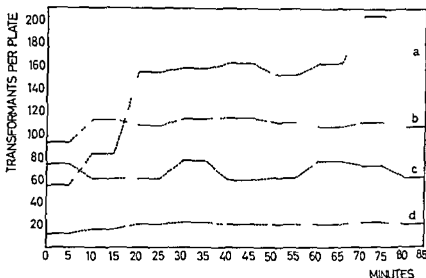


Fig 4

Transformability at 32–33° C following storage at —20° C 0 at the abscissa indicates 15 min after removal from —20° C —— 5 min DNA exposure periods ---- Intervals not examined a *Moraxella nonliquefaciens* 4663/62, 4 days storage b *Moraxella nonliquefaciens* 7784, 7 days storage c *Neisseria catarrhalis* Ne 11 5 days storage d *Moraxella bovis* 10900, 2 days storage

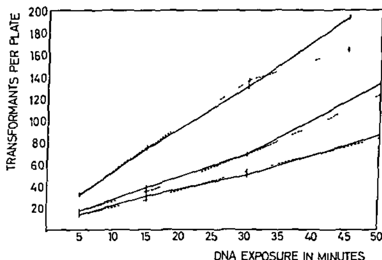


Fig 5

Variation of transformant yield with the duration of DNA exposure The transformations are started at 40 min after removal of recipients from —20° C Upper set of curves Homologous transformation of *Neisseria catarrhalis* Ne 11 Middle set of curves Homologous transformation of *Moraxella nonliquefaciens* 4663 62 Lower set of curves Heterologous transformation of *Moraxella nonliquefaciens* 4663 62 with *Moraxella bovis* 10900 DNA The middle and lower set of curves represent simultaneous experiments Stippled lines Lowest DNA concentrations for saturation control (20 µg per ml in the upper experiment 10 µg per ml in the two others) Continuous lines Highest total DNA concentration (approximately 40 µg per ml)

cannot provide an accurate measurement of cell number Hence assay for recipient cells is subject to systematic errors On the other hand, the occurrence of more than one transformation per aggregate must be relatively infrequent Thus, assay for transformed colonies most often very nearly indicate the number of transformed cells The exact calculation of the fractional frequency of transformation is therefore difficult, whereas comparisons between frequencies of transformation *in aliquots of the same recipient population* as a rule are considered valid (in part literally cited from *Lerman & Tolmach* 1957, p 70) A reservation on this point has to be made In cases of pronounced aggregation, which might occur in some *Neisseria* recipient, for instance, the ratio between heterologous and homologous transformation may be estimated too high This assumption is based on the likelihood that the frequency of multiple homologous transformations of a large aggregate is greater than the probability of multiple heterologous "hits"

Assay for recipient cells, which is of minor importance in comparative transformation, is undertaken on blood agar surface Transformant scores are regularly means of 3 or more parallel plate counts The method of surface counting of transformants is characterized by the following estimation of the standard deviation of counts, based on 79 separate series of 3 observations each With means between 20 and 50 colonies per plate $\sigma = 4\ 4$, between 51 and 100 colonies $\sigma = 8\ 1$, between 101 and 150 colonies $\sigma = 11\ 7$, and between 151 and 250 colonies $\sigma = 16\ 4$

ADDITIONAL REMARKS

It is beyond the scope of this paper to make a complete report on the mechanisms of transformation An excellent review on these subjects has been presented by *Ravin* (1961)

The rational basis of using transformation and other types of genetic transfer in the study of relationships between *microorganisms* has been discussed by *Ravin* (1960) and *Lanni* (1960) *Quantitative transformation* procedures for this purpose are fundamented on the *assumption that* uptake of DNA is non specific, *i e* that DNAs from homologous *and heterologous* donors are made inaccessible to deoxyribonuclease at identical rates Thus differences between heterologous and homologous *transformation* frequencies are considered to reside in the chromosomal pairing and integration (*Schaeffer* 1958)

The non specificity of DNA uptake has been shown in pneumococci by *Lerman & Tolmach* (1957) They found that ^{32}P labelled *E coli* and pneumococcal DNAs were incorporated by competent pneumococci with almost equal efficiency Lack of ut [illegible]

[illegible] DNA from the closely related strain Challis, from

for 50 min, after an initially sharper rise of counts of transformants. Whether transformation frequencies are compared at 5, 15, 30 or 50 min does not influence the ratio of heterologous to homologous transformation, which varies between 1.7 and 2.0 $\cdot 10^{-3}$ (calculations not directly available from the figure).

The strain *Moraxella nonliquefaciens* 7784 seems to behave differently in transformation experiments according to this procedure. Fig. 6 illustrates the transformation of this strain with DNA from *Moraxella bovis* 10900. There is an increase of transformants, linearly with time for 25–30 min, whereafter a plateau is reached. In supplementary experiments with the same recipient, transformants in both homologous and heterologous transformation were scored after 30, 45, 60, 75 and 90 min. of DNA exposure (Fig. 6). There is observed a continuation of the plateau of the former experiment, with a rise towards the end of the observation period at 90 min. The ratio of heterologous to homologous transformation varies between 1.5 and 1.7 $\cdot 10^{-3}$ (calculations not directly available from the figure).

The early linear increase of transformants with the duration of DNA exposure has been observed by *Fox & Hotchkiss* (1957) in pneumococcal transformation and by *Goodgal & Herriott* (1961) in the transformation of *Haemophilus*. The results of the present experiments do not indicate that the duration of DNA exposure is a factor of importance for the determination of ratios of heterologous to homologous transformation frequencies. Similar results have been obtained by *Schaeffer* (1956), studying heterologous transformation in *Haemophilus*.

A DNA exposure time of 30 min. may be considered dangerously long when studying the data of Figs. 5 and 6, for the following reasons. DNA saturation may be easier for practical purposes when a shorter exposure is applied. Secondly, the continuous rise of transformant count after 30 min. in some strains, as compared with a plateau of considerable duration in another strain, may be inherent with variations of receptivity in connection with "pregrowth" or growth phenomena from this time on in some strains (see also Fig. 4). Therefore, it is considered most safe to begin transformation 25 min. after the initial temperature adjustment and apply a 15 min. exposure period. When performing comparisons between only a couple of donors, it might be safe to expose for more than 15 min. even in instable strains, if the difference in time between the first and the last parallel is only a couple of minutes and if DNA saturation is under satisfactory control. This may be useful when transformability is low.

Cell Assays

In these bacteria and especially in *Neisseria*, aggregates in fluid media are unavoidable and variation in the number of cells per colony center must be expected. Therefore, the assay for total colony centers

cannot provide an accurate measurement of cell number. Hence assay for recipient cells is subject to systematic errors. On the other hand, the occurrence of more than one transformation per aggregate must be relatively infrequent. Thus, assay for transformed colonies most often very nearly indicate the number of transformed cells. The exact calculation of the fractional frequency of transformation is therefore difficult, whereas comparisons between frequencies of transformation in aliquots of the same recipient population as a rule are considered valid (in part literally cited from *Lerman & Tolmach* 1957, p. 70). A reservation on this point has to be made. In cases of pronounced aggregation, which might occur in some *Neisseria* recipient, for instance, the ratio between heterologous and homologous transformation may be estimated too high. This assumption is based on the likelihood that the frequency of multiple homologous transformations of a large aggregate is greater than the probability of multiple heterologous "hits".

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The non-specificity of DNA uptake has been shown in pneumococci by *Lerman & Tolmach* (1957). They found that ^{32}P-labelled *E. coli* and pneumococcal DNAs were incorporated by competent pneumococci with almost equal efficiency. Lack of uptake specificity also seems to exist in the transformation of streptococci. *Pakula, Hulanicka & Walczak* (1960) found that competent cells of *Streptococcus sbe* did not discriminate between DNA from the closely related strain Challis, from

for 50 min., after an initially sharper rise of counts of transformants. Whether transformation frequencies are compared at 5, 15, 30 or 50 min. does not influence the ratio of heterologous to homologous transformation, which varies between 1.7 and $2.0 \cdot 10^{-3}$ (calculations not directly available from the figure).

The strain *Moraxella nonliquefaciens* 7784 seems to behave differently in transformation experiments according to this procedure. Fig. 6 illustrates the transformation of this strain with DNA from *Moraxella bovis* 10900. There is an increase of transformants, linearly with time for 25–30 min., whereafter a plateau is reached. In supplementary experiments with the same recipient, transformants in both homologous and heterologous transformation were scored after 30, 45, 60, 75 and 90 min. of DNA exposure (Fig. 6). There is observed a continuation of the plateau of the former experiment, with a rise towards the end of the observation period at 90 min. The ratio of heterologous to homologous transformation varies between 1.5 and $1.7 \cdot 10^{-3}$ (calculations not directly available from the figure).

The early linear increase of transformants with the duration of DNA exposure has been observed by *Fox & Hotchkiss* (1957) in pneumococcal transformation and by *Goodgal & Herriott* (1961) in the transformation of *Haemophilus*. The results of the present experiments do not indicate that the duration of DNA exposure is a factor of importance for the determination of ratios of heterologous to homologous transformation frequencies. Similar results have been obtained by *Schaeffer* (1956), studying heterologous transformation in *Haemophilus*.

A DNA exposure time of 30 min. may be considered dangerously long when studying the data of Figs. 5 and 6, for the following reasons: DNA saturation may be easier for practical purposes when a shorter exposure is applied. Secondly, the continuous rise of transformant count after 30 min. in some strains, as compared with a plateau of considerable duration in another strain, may be inherent with variations of receptivity in connection with "pregrowth" or growth phenomena from this time on in some strains (see also Fig. 4). Therefore, it is considered most safe to begin transformation 25 min. after the initial temperature adjustment and apply a 15 min. exposure period. When performing comparisons between only a couple of donors, it might be safe to expose for more than 15 min. even in instable strains, if the difference in time between the first and the last parallel is only a couple of minutes and if DNA saturation is under satisfactory control. This may be useful when transformability is low.

Cell Assays

In these bacteria and especially in *Neisseria*, aggregates in fluid media are unavoidable and variation in the number of cells per colony center must be expected. Therefore, the assay for total colony centers

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In *Haemophilus*, the non-specificity of DNA uptake has been shown in some species intertransformable at different rates However, contrary to the findings in streptococcal transformation, *Haemophilus influenzae* discriminates between DNAs from closely related species and other DNAs in the comparative inhibition of transformation (*Schaeffer* 1958, *Schaeffer, Edgar & Rolfe* 1960) Therefore, it is theoretically possible that low heterologous to homologous transformation ratios are not always explained exclusively through pairing and integration Unknown DNA uptake specificities should also be considered

Excluding differential factors acting before and during DNA uptake, the question still remains whether streptomycin resistance transformation ratios are representative for general genotypic affinities This is a subject for later investigation and discussion

The most important advantages of the method presented are considered to be its simplicity and the selection of transformants in surface culture The former allows large-scale work with minimal requirements for equipment and technical assistance, and the latter provides among other advantages an easy control of transformants The order of accuracy of the method is considered satisfactory for quantitative determinations

SUMMARY

The development of a method for the application in quantitative transformation to streptomycin resistance of *Moraxella* and *Neisseria*, is described Various aspects of the method are discussed in relation to experimental data presented

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The present report gives the results of immunoelectrophoretic analysis of sterile sera inoculated with bacteria isolated from two of these sera

MATERIAL AND METHODS

Samples from two contaminated sera were cultivated by conventional aerobic methods and the following bacteria isolated

swarming

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mple I)

n sample II)
from sample II)

—

For some experiments other strains from the bacterial collection of the Department of Diagnostic Bacteriology, Statens Seruminstitut were used

Serratia marcescens S 111
Pseudomonas maltophilia AB 942
Pseudomonas maltophilia AB 1263
Aeromonas hydrophila AB 1264

contain approximately 10^9 o
suspensions was added to 1
centration of 5×10^5 organisms per ml of serum The tubes were kept at room
tem ... of tubes was kept at 4° C. As controls unconta
mi

tio

75×10^9 organisms per ml (optically) Duplicate mixtures in stoppe ... were made from *equal parts* of these suspensions and serum Serum/saline and bacterium saline mixtures were used *as controls* The tubes were kept at room temperature and at 37° C (waterbath) and examined *after 2 3 hours 24 hours* and in a few cases later

Immunoelectrophoretic analysis of the *supernatant of the centrifuged specimens* was performed as described earlier (12 14) The *duration* of the electrophoretic separation was 2 hours and the voltage 6 to 7 volts per cm Diffusion took place at 37° C (at the Univ *Inst of Forensic Med*) or at room temperature (at Statens Seruminstitut) for about *24 hours Polyvalent* rabbit anti human sera containing antibodies against the Gc proteins were used for the development of precipitates

Preliminary *reading of the results* was carried out on the native preparations Further analysis of the patterns, however was based upon the amido black stained preparations and the film negatives of the native preparations

RESULTS

In *Experiment I* it was intended to simulate "natural contamination of serum and consequently *relatively* small amounts of bacteria were used

In the samples kept at 4° C no visible growth occurred and no

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The University Institute of Forensic Medicine, Department of Serology (Head: K. Henningsen, M.D.), Copenhagen, and Statens Seruminstitut, Department of Biophysics (Head: A. Birch Andersen, M.Sc.) and Department of Diagnostic Bacteriology (*Head: H. Lautrop, M.D.*), Copenhagen

ALTERATION OF THE Gc PATTERNS IN HUMAN SERA INCUBATED WITH BACTERIA[1]

By

B. Nerstrøm, B. Mansa and W. Frederiksen

Received 9.xii.63

On the basis of a polymorphism within the α-2-globulin region, described by *Hirschfeld* (5) in 1959, it is possible to classify human sera as belonging to one of three distinct phenotypes Gc 1–1, Gc 2–1 and Gc 2–2. Comprehensive family and mother-child studies have shown that the variation of the Gc proteins are genetically determined by two autosomal alleles, Gc^1 and Gc^2, with no dominance, (7, 2, 3, 1, 4, 6, 8, 9, 18, 14, 15). So far a change from one Gc phenotype to another in an individual has never been observed. The Gc groups in serum are found to be widely unaffected by evaporation, prolonged storage at −20° C, and repeated freezing and thawing.

In recent experiments, however, it was demonstrated that exogenous factors may modify the Gc phenotype (16, 17). An enzyme like factor which was released from leucocytes and thrombocytes by freezing and thawing transformed the Gc proteins into an α-1 globulin immunologically closely related to or identical with the Gc proteins. A similar transformation was induced by *Crystalline Bacterial Proteinase Novo* prepared from a subtilis strain.

The present study intends to present evidence that deviating Gc patterns may also be due to contamination of the sera with bacteria. The study was initiated by the observation of immunoelectrophoretic changes ranging from slightly deviating Gc patterns to severe deformation of the whole pattern in human sera which appeared to be contaminated (13, 14, 15). In a preliminary experiment (unpublished results) it was found that in samples withdrawn with no special precautions to avoid contamination capillary blood displayed deterioration of the immunoelectrophoretic pattern earlier than blood withdrawn by venepuncture when stored at room temperature. Samples from five of the sera, which displayed severely deformed patterns, were cultivated and shown to be grossly contaminated with several different bacteria.

1 This study was supported by a grant from the foundation *Kong Christian den Tiendes Fond*.



Fig 1
Immunoelectrophoretic patterns of serum inoculated with different bacteria and incubated at room temperature

veeks
or 4 weeks

Gc precipitates were observed after 18 days In this case the electrophoretic position of the Gc components was not affected, but the distance between the Gc precipitates and the antibody trough increased gradually indicating a decrease in concentration of the antigens The concentration of the Gc 2 component appeared to decrease at a higher rate than that of the Gc 1 component Concomitantly with this reduction in concentration of the normal Gc proteins a new precipitate appeared in the α-1 globulin region This precipitate again showed reaction of immunological identity with the Gc proteins and the electrophoretic position of the precipitate was apparently the same in sera of type Gc 1 1 Gc 2 1 and Gc 2 2 The immunological relation to the Gc proteins was readily seen in Gc 1 1 and Gc 2 1 sera and might be established in Gc 2 2 sera by admixture of normal Gc 1–1 serum prior to electrophoresis The changes of the Gc patterns increased during prolonged storage and finally the new α 1 precipitate as well as the normal Gc precipitates disappeared completely whereas the precipitates of several other components remained unchanged

The differences between the Gc changes induced by *Serratia marcescens* and *Pseudomonas maltophilia* are illustrated in Fig 1

As far as the other serum proteins are concerned it must be stressed that the first recognizable change of the immunoelectrophoretic patterns

alteration of the immunoelectrophoretic patterns was observed within 40 days

In the sera stored at room temperature macroscopically visible growth appeared in the tubes with *Serratia marcescens* and *Pseudomonas maltophilia* within the first one to two weeks The remaining five bacteria gave only rise to insignificant growth during the experimental period The results of the immunoelectrophoretic analysis are summarized in Table 1.

TABLE 1

Changes of the Gc Precipitates in Sera Incubated with Different Bacteria at Room Temperature

Bacteria added	Gc group of serum	Duration of incubation in days						
		6	11	15	18	28	35	42
No bacteria	Gc 1-1	0	0	0	0	0	0	n d
	Gc 2-2	0	0	0	0	0	0	n d
	Gc 2-1	0	0	0	0	0	0	n d
S marcescens	Gc 1-1	0	$(\alpha_1)\leftarrow$	–	–	–	–	n d
	Gc 2-2	0	$\leftarrow$	$(\alpha_1)\leftarrow$	–	–	–	n d
	Gc 2-1	0	$(\leftarrow)$	$\leftarrow$	$(\alpha_1)\leftarrow$	–	–	n d
Ps maltophilia	Gc 1-1	0	0	0	$(\uparrow)$	$\alpha_1 \uparrow$	$\uparrow$	–
	Gc 2-2	0	0	0	$(\uparrow)$	$\alpha_1 \uparrow$	$\leftarrow\uparrow$	–
	Gc 2-1	0	0	0	$(\uparrow)$	$\alpha_1 \uparrow$	$\uparrow$	–
Remaining 5 bacteria (2, 3, 4, 5 6)	Gc 1-1	0	0	0	0	*)	*)	n d
	Gc 2-2	0	0	0	0	0	0	n d
	Gc 2-1	0	0	0	0	0	*)	n d

0 normal Gc precipitates
$\leftarrow$ displacement of Gc precipitates towards the anode
$\uparrow$ displacement of Gc precipitates away from the trough
– complete disappearance of Gc precipitates
α_1 occurrence of a new α 1 globulin, immunologically identical with Gc
* insignificant changes
n d not done

The uncontaminated control sera gave quite normal immunoelectrophoretic patterns during the experimental period

In the three sera incubated with *Serratia marcescens* the Gc precipitate was displaced anodically from the 11th day The displacement towards the anode increased during prolonged storage, whereas the shape of the precipitates did not change Later in the incubation period, however, the precipitates became weaker and an anodic extension of the precipitate or a faintly developed new α-1-precipitate, immunologically identical with Gc, occurred Finally the Gc precipitates disappeared from the patterns at a time when the majority of the serum proteins displayed little or no signs of deterioration The change was essentially the same in all three types of sera

In the sera incubated with *Pseudomonas maltophilia* changes of the

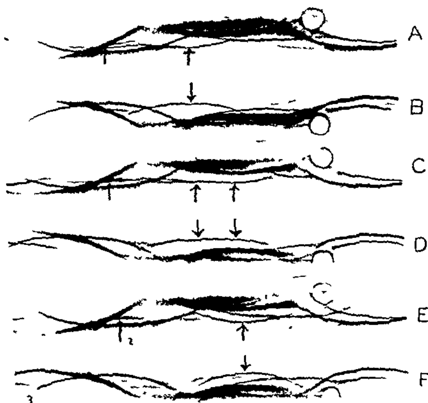


Fig 3

Immunoelectrophoretic patterns of serum of type Gc 1-1 (*A*), Gc 2-1 (*C*), and Gc 2 2 (E) after 24 hours of incubation at 37° C with big doses of *Pseudomonas maltophilia* (AB 942) Sterile reference sera of type Gc 1 1 (*B*), Gc 2-1 (*D*), and Gc 2 2 (*F*) Antiserum R 1071

TABLE 2

The Effect of Gc Proteins of Different Strains of some Bacterial Species
(Experiment II)

Bacterial strain	*Observed changes of Gc precipitates*
Serratia marcescens (sample I) *Serratia marcescens* (S 111)	Anodic displacement
Serratia alba (sample II) *Cytophaga anitrata* (sample II)	No changes
Pseudomonas maltophilia (sample II) *Pseudomonas maltophilia* (AB 1263) *Pseudomonas maltophilia* (AB 942) *Aeromonas hydrophila* (AB 1264)	Transformation into a new α 1 globulin precipitate

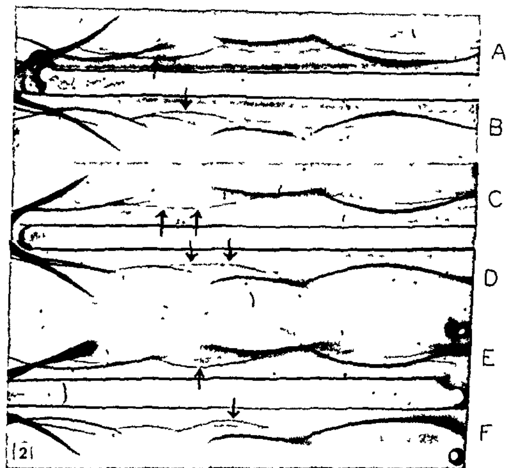


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was a cathodic displacement of α-1-glycoprotein (3 5 S) This displacement occurred 5 to 7 days before the Gc changes Anodic displacement of the α-2-macroglobulin usually occurred later during the incubation than the Gc changes The albumin and transferrin precipitates were not displaced during the experimental period

Concerning the remaining five bacteria (numbered 2, 3, 4, 5, and 6 in Table 1), no convincing changes occurred within 4 to 5 weeks All samples were re-examined after 54 days and now almost every sample displayed one or more of the alterations described However, a control culture at this time from each test tube revealed that many tubes were now contaminated with other bacteria These late results were therefore discarded and further examination on the possible occurrence of changes after prolonged storage with these bacteria was not performed

In *Experiment II* different strains of *Serratia marcescens*, *Pseudomonas maltophilia* and a few other species were examined using greater

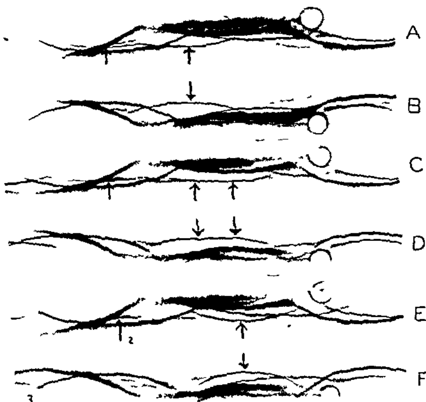


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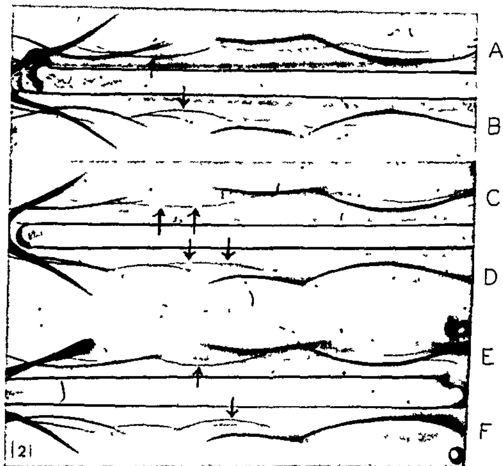


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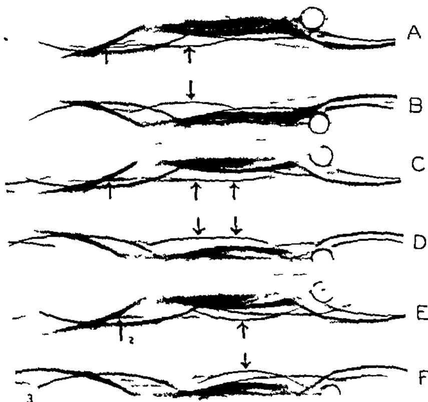


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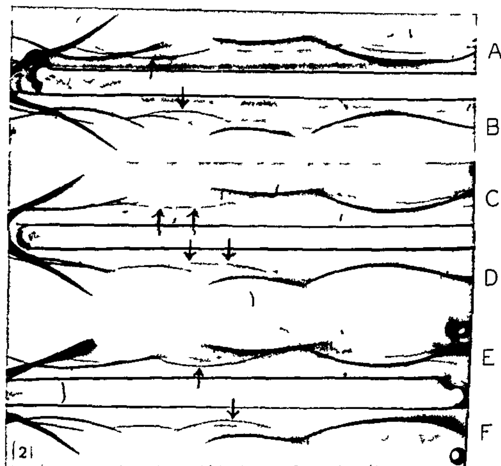


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In *Experiment II* different strains of *Serratia marcescens Pseudomonas maltophilia* and a few other species were examined using greater

Serratia marcescens induced a gradual increase in electrophoretic migration of the Gc components while the shape of the precipitates remained unchanged for some time The interpretation of this may be that each of the Gc molecules in the serum was subjected to the same minute and gradually occurring alterations

Pseudomonas maltophilia and *Aeromonas hydrophila* induced a change which appears to be identical with the change provoked by disintegrated leucocytes and thrombocytes and by *Crystalline Bacterial Proteinase Novo* (16, 17) This change is interpreted as a successive transformation of the two normal Gc components into a faster migrating component immunologically identical with Gc proteins

At the present time the reactions responsible for the two different Gc transformations are unknown, but they are most probably of proteolytic nature

The importance of these alterations of the Gc patterns is obvious, as the classification of sera in different Gc groups is based upon the differences in electrophoretic mobility The experiments with *Serratia marcescens* show that contaminated Gc 2 2 serum after a certain period may present a Gc precipitate indistinguishable from a Gc 1–1 precipitate (see Fig 2) However, judging from extensive family, mother child, and twin investigations, the alterations of the Gc patterns due to *contamination will usually be recognizable* due to other alterations induced simultaneously So far, one or more of the signs of deterioration has always been present in our contaminated sera Asymmetry of the precipitate anodic elongation a new α 1 precipitate, reduction of concentration (as compared to the other fractions), or displacement of some other precipitates, most frequently of the α 1 glycoprotein

However with certain antisera, for instance antisera lacking anti α 1 glycoprotein the Gc changes may be the only demonstrable changes in the patterns This fact stresses the importance of using polyvalent antisera and further that deviating Gc patterns must always be verified on new samples of good quality

SUMMARY

Changes of the Gc patterns in normal human sera are developed after incubation with *Serratia marcescens, Pseudomonas maltophilia* and *Aeromonas hydrophila* but not with a series of other bacterial species isolated from two stored serum samples obtained from capillary blood *Serratia marcescens* induces a steadily increasing mobility of the Gc proteins *Pseudomonas maltophilia* and *Aeromonas hydrophila* induce a transformation of the Gc proteins into a component immunologically identical with Gc and apparently with the same position in the α 1-globulin region The changes are assumed to be provoked by proteolytic enzymes produced by the bacteria The results are discussed with a special reference to the reliability of Gc grouping in genetical work

amounts of bacteria and higher incubation temperature The experiment was performed independently at two different laboratories with the use of a series of different polyvalent rabbit anti-human sera

The Gc changes occurred within 2 to 24 hours as compared to 11 to 18 days in the first experiment The results of the two different laboratories were in complete agreement and qualitatively confirmed the results obtained in Experiment I (Figs. 2 and 3). The results are summarized in Table 2.

In one single case a slight incongruity was observed between the alterations induced by different strains of the same species One *Pseudomonas maltophilia* strain (AB 1263) reduced the concentration of the Gc-2 component at a higher rate than the concentration of the Gc-1 component, as did the strain originally isolated from an accidentally contaminated serum sample (Fig 1). The other strain (AB 942) reduced the concentration of both Gc components equally, so that the Gc 2–1 precipitates remained symmetric until they disappeared from the patterns (Fig 3)

DISCUSSION

Previously it has been shown that the electrophoretic mobility of some serum glycoproteins may be changed by incubation with certain bacteria and bacterial filtrates (20, 10, 11) Usually the change is a retardation (*i e* cathodic displacement) which is ascribed to the action of neuraminidases which split sialic acids from the prosthetic groups

In the present investigation two out of seven bacterial species, isolated from accidentally contaminated blood, and one more species of different origin were capable of inducing changes in the immunoelectrophoretic patterns of serum The three bacterial species were *Serratia marcescens*, *Pseudomonas maltophilia* and *Aeromonas hydrophila*, all being strongly proteolytic when tested by conventional methods With the antisera used the recognizable changes comprised α-1 glycoprotein (3 5 S), the group-specific components (Gc) and α-2 macroglobulin The electrophoretic position of albumin and of transferrin was not affected

The retardation of α 1-glycoprotein (3 5 S) as observed in the present experiments agrees with the former observation (20) and might thus be due to a neuraminidase activity of the bacteria Against this assumption is the fact that other glycoproteins containing sialic acid such as the transferrin, were not affected At present we have no explanation to offer on this problem

Concerning the Gc proteins a neuraminidase activity cannot be responsible for the observed changes *Schultze et al* (19) did not find any neuraminic acid in purified Gc proteins Furthermore, they demonstrated that the mobility of the Gc proteins is not affected by neuraminidase. Thus the Gc changes demonstrated in the present experiments seem to be of a more complex nature

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Statens Seruminstitut, Amager Boulevard 80, Copenhagen S.
Streptococcal Department (Chief Knud Skadhauge, M D).

RADIATION RESISTANCE OF ENTEROCOCCI DRIED IN AIR

By

EBBE AHRENSBURG CHRISTENSEN

Received 14 xii 63

In the course of experiments on the radiation resistance of dried preparations of various bacteria isolated from dust and soil, two strains of Gram positive, oval cocci were found, growing in diplo-form or in short chains of three or four Dried in air, these vegetative bacteria have survived electron irradiation with 2 0 Mrad Both strains were *Streptococcus faecium* As the radiation resistance of these strains was of the same order of magnitude as the resistance of bacterial spores, and might therefore be of significance in the radiation sterilization of disposable equipment for use in hospitals and in medical practice, a number of bacterial strains all belonging to the enterococcal group were examined for their resistance to ionizing radiation

The enterococci have previously been reported to have a considerable resistance to ionizing radiation, in comparison with the majority of vegetative bacteria (2, 6, 7, 10, 12, 13) The number of strains studied, however, does not appear to be very great

MATERIAL AND METHODS

The following strains of enterococci were studied 19 laboratory strains belonging to *Streptococcus faecium* 12 laboratory strains belonging to *Str. faecalis*, 2 laboratory strains belonging to *Str durans*, and 5 newly isolated strains, all belonging to *Str faecium* [1]

The bacteria were grown on blood agar for two days at 37° C The culture was scraped from the plates suspended in serum broth (3) and dried as drops (0 01 ml) on polyethylene foil in atmospheric air Before and after irradiation, the number of viable units per test piece was determined by suspension in phosphate buffered saline pH 7 38 and by usual dilution and counting technique All counting of viable units was performed on blood agar

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[1] The author wishes to thank *Knud Skadhauge* M D and *Ebbe Kjems* M D both of Statens Seruminstitut for identifying the newly isolated strains and for making the laboratory strains available

[2] The author wishes to thank *the Danish Atomic Energy Commission* and the staffs of the accelerator and Co⁶⁰ facilities

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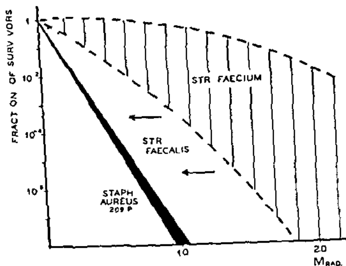


Fig 2
Inactivation of enterococci by irradiation

DISCUSSION

It is well known that the resistance of bacteria to ionizing radiation can vary considerably with the environmental conditions prior to, during and after the irradiation (5) Bacterial spores are generally more resistant than vegetative bacteria, but a few strains of Gram-positive cocci are known to be far more resistant to irradiation than other micro-organisms even though these micrococci are not spore-forming organisms (1, 3 4, 9)

As mentioned it was observed earlier that enterococci could be relatively radiation resistant However, it seems not to have been reported that on a dose level commonly used in radiation sterilization of surgical materials some enterococcal strains in the dried state could be more resistant than the most radiation resistant among those bacterial spores common in airborne dust In the present study, the radiation resistance of the bacteria was examined using crude bacterial preparations dried and stored in atmospheric air at ordinary room temperature as the resistance under these circumstances must be considered to be a useful approximation to the resistance of the bacteria in routine sterilization of dry plastics

Whether faecium strains show a correspondingly high radiation resistance in aqueous suspension has so far not been investigated

Powell & Bridges (12) assume that as sterilizing dose, 2 5 Mrad should provide adequate safety under most conditions This dose will give an inactivation factor of about 10^4 for the most resistant of the faecium strains which have been used in the present study in con

RESULTS

The majority of the strains were irradiated with two doses only, 1 5 and 2 0 Mrad, but a few strains were examined in four independent *irradiation experiments with doses from 0 3 to 4 5 Mrad, and with three different preparations of test pieces* The inactivation curves for these strains are known over eight logarithmic steps The inactivation curves are non-linear (Fig 1)

All the faecium strains studied were more resistant than the other enterococcal strains, but there was very considerable variation in resistance among the faecium strains (Fig 2) At 2 0 Mrad, 15 of a total of 24 strains belonging to *Str faecium* were more resistant to irradiation than the most resistant bacterial spores (*B subtilis, B globigii, B pumilus*) examined in the same laboratory by a similar technique (3) (Fig 1) At 3 0 Mrad at least three of the strains were more resistant than the above mentioned spores

The resistance of the strains to drying and storage in the dried state in atmospheric air was found to vary, but under the conditions mentioned, test pieces with the most radiation-resistant strain were found to have an unchanged number of viable units and unchanged radiation resistance after being stored for 8 weeks

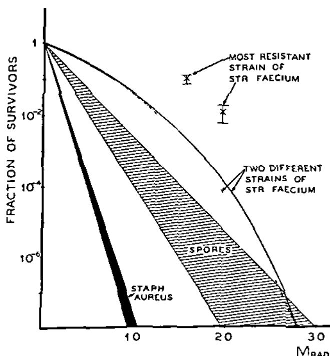


Fig 1

Inactivation curves for dried bacteria and bacterial spores examined by the same technique

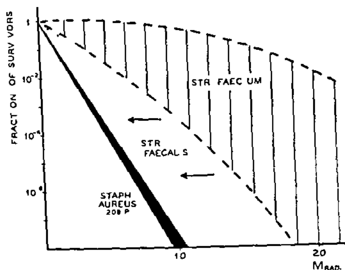


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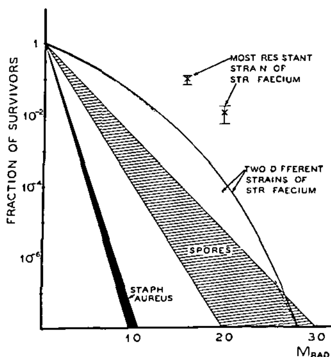


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Inactivation curves for dried bacteria and bacterial spores examined by the same technique

Acta path et microbiol scandinav 61, 487–490, 1964

Pneumococcus Department, Statens Seruminstitut, Copenhagen

DISTRIBUTION OF AGE AND SEX IN CASES OF PNEUMOCOCCAL MENINGITIS

By

Erna Lund

Received 16 xii 63

During a sixteen-year period, 1947–1962, 669 cerebrospinal fluids submitted to Statens Seruminstitut for examination were found to contain pneumococci, *i e* an average of 42 positives a year All pneumococci have been typed and 38 of the 46 known *Pneumococcus* types or groups have been identified As the eight types not found (pn 37, 39, 42, 43, 45, 46, 47, 48) are among those rarely seen, it seems likely, however, that all pneumococcal types occasionally may occur in the cerebrospinal fluids In consequence, sera against all known types should be available for identification of isolated strains (*Lund* 1963)

Table 1 shows the frequency of the various *Pneumococcus* types found in cases of meningitis during the 16-year-period 1947–1962 The ten most common types were 3, 18, 1, 6, 7, 14, 19, 2, 12, 9, the frequency of the types varying from year to year The types found most often in a single year were the following type 1 (prevalent in 3 years), 3 (2 years), 4 (1 year), 6 (2 years), 7 (3 years), 12 (3 years), 14 (2 years), 18 (3 years), 23 (1 year)

Table 1 also shows the distribution within the following age groups: less than 2 years, 2–9, 10–19 etc in groups of 10 years Out of 669 patients, 307 (46 per cent) were under 10 years, and 342 (51 per cent) more than 10 years, so practically half the patients were children and half adults Above the age of ten, the number of patients was almost the same (40–66) within each 10 year age group

The oldest patient was 81 years old and the youngest one month, but all age groups were represented

Out of the 669 patients, 397 (59 per cent) were males, 266 (40 per cent) females The sex of 6 patients has not been recorded Thus the frequency in males and females is of the order 3 2 The same proportion was found earlier by *Gregersen* (1961) and *Quaade & Kristensen* (1961) Table 2 shows the frequency in males under 10 years to be 29 per cent, in males over 10 years 28 per cent In females under 10 years 16 per cent and in females over 10 years 23 per cent Under the age of 10, the number of meningitis cases is thus almost twice as high in boys as in girls

ditions resembling those in radiation sterilization of disposable equipment for hospital use This factor appears to be rather low in relation to the norms usually recommended for sterilization by autoclaving (8, 11)

SUMMARY

The radiation resistance of a total of 38 strains of enterococci was determined by means of gamma and electron irradiation

24 *strains belonging to Streptococcus faecium* were more resistant than the remaining 14 strains belonging to *Streptococcus faecalis* and *Streptococcus durans* Irradiated with doses in common use for radiation sterilization, at least three of the faecium strains examined in the dry state were more resistant than subtilis and pumilus spores examined by the same technique

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often When all children less than 10 years old were grouped together, the most frequent types were 18, 6, 14, 7, 19 In individuals 10–40 years old no particular type was prevalent

TABLE 2

Distribution of Sex and Age ($\lesseqgtr$ 10 Years) in 669 Patients with Pneumococcal Menigitis (1947–62)

Year	♂			♀			♂ or ♀		Year	♂			♀			♂ or ♀	
	< 10	> 10	age*	< 10	> 10	age*	< 10	> 10		< 10	> 10	age*	< 10	> 10	age*	< 10	> 10
1947	18	13	2	8	10	2			1955	12	11		6	11		1	
1948	12	10	1	10	13	2			1956	11	4		7	5	1		
1949	9	12	4	8	8	1			1957	16	18	1	3	10	1		
1950	10	10		8	9				1958	16	8		3	9			
1951	8	12		7	10	1	1		1959	12	11	1	7	9			
1952	13	19	1	5	12			1	1960	12	15		8	9		1	
1953	7	11		7	6				1961	17	10		9	7			
1954	11	10		6	6				1962	13	14	2	4	18		1	1
									number	197	188	12	106	152	8	4	2
									per cent	29	28	2	16	23	1	1	

TABLE 3

Age Distribution among 307 Children below the Age of 10 Years Presenting Pneumococcal Meningitis

Children 10 years	Years of age				
	2	2 3	4 5	6 7	8 9
Number	192	24	36	26	29
Per cent	63	8	12	8	9

TABLE 4

Frequency of Pneumococcus Types Causing Meningitis in 200 Patients > 40 Years

Type or group	♂	♀	♂ + ♀	Type or group	♂	♀	♂ + ♀
1	23	8	31	19	9	2	11
2	6	4	10	20	1		1
3	18	20	38	22	2	3	5
4	8	2	10	23		3	3
5		2	2	24		1	1
6	1	4	5	25	2	1	3
7	5	12	17	27		1	1
8	6	6	12	32	1		1
9	2	2	4	33	1	4	5
11	1	2	3	34	2		2
12	6	5	11	35	2		2
14	1	5	6	36	1		1
16	1	1	2	38		2	2
17		2	2	41	1		1
18	4	4	8				
				number	104	96	200
				per cent	52	48	

The types omitted were not isolated

TABLE 1

Frequency of Pneumococcus Types (1947 to 1962) in Meningitis at Different Ages

Type	Age in years									Unknown age	Total	%
	< 2	2–9	10–19	20–29	30–39	40–49	50–59	60–69	> 70			
1	6	10	7	5	8	8	10	2	1	4	61	9
2	11	1	1	3	4	4	4	1	1	1	30	4
3	4	6	7	1	4	10	10	12	6	4	64	10
4	6	7	1		1	2	4	2	2		25	4
5	4		2	1	1	1		1			10	1
6	27	11	6	4	4	1	2	2			57	9
7	13	11	2	1	2	3	5	5	4	3	55	8
8	2	5	3		1	3	5	3	1		23	3
9	12	6	1		4	2	1		1		27	4
10	4	3	2		1						10	1
11					1	1		1	1		4	1
12	6		2	3	6	3	5	3			28	4
13		1	2	1	1						5	1
14	28	7	2		2		1	5		2	47	7
15	5		3	1	1					1	11	2
16			2		1		1		1		5	1
17	2			1		1		1			[illegible]	1
18	21	26	2	2	2	2	2	5		2	64	10
19	16	11		1	2	4	2	3	2	1	42	6
20		1		1			1				3	
21		1		1	1					1	4	1
22	2	1	2	1			2	1	1	1	11	2
23	12	4	1	3	2	1	1		2		26	4
24	1	1	3		2		1				8	1
25					3		3				6	1
27	2							1			3	
28	1										1	
29				1							1	
31				1							1	
32			1			1					2	
33		2		1		1	3	1			8	1
34			2	4	1		1		1		9	1
35			1	1		1			1		4	1
36							1				1	
38			1	1		1		1			4	1
40	1			1							2	
41							1				1	
44			1								1	
Total	192	115	57	40	54	50	66	50	25	20	669	
Total	307		342							20		
%	46		51							3		

In Table 3 the 307 children less than 10 years old have been divided into 2-year age groups and 192 that is 63 per cent of the children and 28 per cent of all patients were found to be under 2 years of age Of these, 120 (62 per cent) were boys and 72 (38 per cent) girls Thus pneumococcal meningitis was most common in the first 2 years of life and again nearly twice as frequent in boys as in girls

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1947	18	13	2	8	10	2			1955	12	11		6	11		1	
1948	12	10	1	10	13	2			1956	11	4		7	5	1		
1949	9	12	4	8	8	1			1957	16	18	1	3	10	1		
1950	10	10		8	9				1958	16	8		3	9			
1951	8	12		7	10	1	1		1959	12	11	1	7	9			
1952	13	19	1	5	12			1	1960	12	15		8	9		1	
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2	6	4	10	20	1		1
3	18	20	38	22	2	3	5
4	8	2	10	23		3	3
5		2	2	24		1	1
6	1	4	5	25	2	1	3
7	5	12	17	27		1	1
8	6	6	12	32	1		1
9	2	2	4	33	1	4	5
11	1	2	3	34	2		2
12	6	5	11	35	2		2
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2	11	1	1	3	4	4	4	1	1	1	30	4
3	4	6	7	1	4	10	10	12	6	4	64	10
4	[illegible]	7	1		1	2	1	2	2		25	4
5	4		2	1	1	1		1			10	1
6	27	11	6	4	4	1	[illegible]	2			57	9
7	19	11	2	1	2	3	[illegible]	5	4	3	55	8
8	2	5	3		1	3	5	3	1		23	3
9	12	6	1		4	2	1		1		27	4
10	4	3	2		1						10	1
11					1	1		1	1		4	1
12	6		2	3	6	3	5	3			28	4
13		1	2	1	1						5	1
14	28	7	2		2		1	5		2	47	7
15	5		3	1	1					1	11	2
16			2		1		1		1		5	1
17	2			1		1		1			5	1
18	21	6	2	2	2	[illegible]	2	5		2	64	10
19	16	11		1	2	4	2	3	2	1	42	6
20		1		1			1				3	
21		1		1	1					1	4	1
22	2	1	2	1			2	1	1	1	11	2
23	12	4	1	3	2	1	1		2		26	4
24	1	1	3		2		1				8	1
25					3		3				6	1
27	2							1			3	
28	1										1	
29				1							1	
31				1							1	
32			1			1					2	
33		2		1		1	3	1			8	1
34			[illegible]	4	1		1		1		9	1
35			1	1		1			1		4	1
36							1				1	
38			1	1		1		1			4	1
40	1			1							2	
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The most common types found in children under 2 years were 14, 6, 18, 7, 1 (Table 1); in the age group 2-9 years type 18 was found most

Acta path et microbiol scandinav 61 491–492, 1964

BRIEF REPORT

RECURRENT CASES OF PNEUMOCOCCAL MENINGITIS

By *Erna Lund*

nce the introduction of chemo

Precechtel 4 (1954) describes 4 cases of recurrent meningitis one patient had 13 attacks *another 6 and two patients two each. Spitze et al* (1961) has seen a boy having meningitis 20 times eleven of these attacks were bacterial six being pneumococcal Finally the boy was operated for a fracture line in the skull, and after that remained healthy These authors think that most cases of recurrent meningitis are preceded by a trauma to the skull and found cerebrospinal fluid otorrhea in nearly 20 per cent of their patients

At Statens Seruminstitut we have recorded 6 cases of recurrent pneumococcal meningitis during the years 1955–1962[1] (Table 1)

TABLE I

6 Cases of Recurrent Pneumococcal Meningitis

No	Sex	Age in years at I	Number of purulent meningitis			Pneumococcal meningitis				
						Type at			Interval in months	
			pn	÷ pn	total	I	II	III	I–II	II–III
1	♀	28	0	2	2	12	12		20	
2	♀	36	0	3	3	12	12	9	6	6
3	♀	49	3	2	5	38	7		35	
4	♂	7	0	2	2	19	34		40	
5	♂	39	1	2	3	19	11		10	
6	♀	17/12	0	2	2	23	23		2	

I (II III) first (second third) attack of pneumococcal meningitis
pn = pneumococci

Some of these patients not only had pneumococcal *meningitis but also one or* more attacks of purulent meningitis of other origin, they had from 2–5 meningeal infections

Of these six patients four were female and two male, two were children (17/12 and 7 years) and four adults (28 36 39, 49 years) One of the patients (no 3) had 5 attacks of purulent meningitis three were of unknown origin and two pneumo

Received 29 iv 64 *from Statens Seruminstitut Pneumococcus Department*

[1] For making their material available I give my best thanks to the following hospitals Blegdamshospitalet Diakonissestiftelsen Odense Amts og Bys Sygehus and Nyborg Sygehus.

TABLE 5

Four most Prevalent Pneumococcus Types in Denmark

Author	Pneum mening	Prevalent types			
Morch (1949)	202	1	3	6	18
Bastrup Madsen & Norby (1955)	30	18	1	6	3
Lund present study (1964)	669	3	18	1	6

Out of 200 patients above the age of 40 years, 104 (52 per cent) were males and 96 (48 per cent) females (Table 4), the numbers thus being almost equal Whilst type 3 was found equally often in both sexes (males 18, females 20), type 1 infections were more frequent in men (23 cases) than in women (8 cases) The most common types among patients above 40 years were type 3 (38 patients), type 1 (31 patients) and type 7 (17 patients)

SUMMARY

At Statens Seruminstitut 669 pneumococci from cases of meningitis have been isolated and typed during a 16-year period (1947–1963) The types most frequently found were 3, 18, 1 and 6 Sixty per cent of the patients were males and forty per cent females Amongst children below the age of 10 years there were almost twice as many boys as girls, but among patients above the age of 40 years the sexes were equally represented

Acta path et microbiol scandinav 61 491 492 1964

BRIEF REPORT

RECURRENT CASES OF PNEUMOCOCCAL MENINGITIS

By *Erna Lund*

... more frequent since the introduction of *chemo-*

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2	♀	36	0	3	3	12	12	9	6	6
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4	♂	7	0	2	2	19	34		40	
5	♂	39	1	2	3	19	11		10	
6	♀	17 12	0	2	2	23	23		2	

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Of these six patients four were female and two male two were children (17/12 and 7 years) and four adults (28 36 39 49 years) One of the patients (no 3) had 5 attacks of purulent meningitis three were of unknown origin and two pneumo

Nyborg Sygehus

coccal Another (no 5) had three attacks, two being pneumococcal (types 19 and 11) and one meningococcal The remaining four patients (nos 1 2 4 6) were infected only by pneumococci two having two attacks and one (no 2) having three

The interval between the attacks was from 2 40 months Two patients (nos 1 and 6) had the same pneumococcal type in both infections (type 12 and type 23) another (no 2) having three attacks had the same type (type 12) in the first two and another (type 9) in the last infection Three patients changed the type (nos 3 4 5) from type 38 to type 7, from type 19 to type 34 and from type 19 to type 11

These six patients had no history of any trauma As mentioned by different authors otorrhea or rhinorrhea may be the source of recurrent infections of the spinal fluid None of these six patients showed an otorrhea, but one (no 3) had a rhinorrhea cerebrospinalis of unknown origin for three years During the following eight years this patient had—as mentioned—five attacks of purulent meningitis Following the last infection X ray showed a fistula through the right lamina cribosa, neuro surgical treatment was recommended, but the patient refused it and was discharged on prophylactic treatment with V penicillin (one tablet of 200 mg daily) Three of the six patients were discharged on prophylactic penicillin (200 mg daily) and during such treatment no meningeal infection took place Two of the patients (nos 1 and 4) became almost totally deaf following their first attack of pneumococcal meningitis

An additional patient (no 7) may be mentioned who had two attacks of purulent meningitis the first of these caused by *Pneumococcus* type 31 the other of unknown origin This patient had a fracture of the skull about 6 months before his pneumococcal meningitis 18 months later he died from his second meningeal infection At autopsy remnants of fracture lines in the skull were observed

With the modern chemotherapy and antibiotic treatment it is possible to cure most patients with a meningitis However if the predisposing condition remains and is associated with acute or chronic infection recurrent attacks of meningitis may occur

When there is communication between the ear or nose to the meninges some authors recommend that this should be closed surgically but prophylactic treatment with oral penicillin seems—at least in some cases—to prevent further meningeal infections

Summary 6 patients with recurrent pneumococcal meningitis are reported Two of these patients had additional meningeal infections of other origin In three cases the *Pneumococcus* type was the same in the first and the second attack but in the remaining cases the type had changed The interval between the pneumococcal meningitis was from 2 40 months Two of the patients became almost totally deaf Prophylactic treatment with penicillin was given successfully to three of the patients

References Cawthorne T E Acta oto laryng 45 429 1954 —*Maxwell J H* Laryngoscope 63 355 1953 —*Nen clus C* Acta oto laryngol 39 314 1951 —*Precechtel, A* Acta oto laryngol 44 427 1954 —*Spit e E B Wagner S Sataloff J Hoffmann N P & Hope J W* J Pediat 59 337 1961

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TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Meeting March 13, 1964,
Stockholm, Sweden

Received 15 iv 64

Lodin A Nystrom B Rosengren S & Willen S Department of Dermatology and Department of Clinical Bacteriology Karolinska Sjukhuset Stockholm *RECURRENCE OF GONORRHOEA PENICILLIN SUSCEPTIBILITY OF GONOCOCCI AND CONCOMITANT PRESENCE OF PENICILLINASE-PRODUCING BACTERIA IN THE URETHRA*

Of 397 outpatients treated for gonorrh ea in 1962 16 or 4 5 per cent of those receiving standard therapy of a single injection of 600 000 units of benzyl procaine penicilling had a true" recurrence of the infect on that could not reasonably be ascribed to reinfection In all these cases the minimum inhibitory concentration of penicillin required by the gonococcal strains isolated was higher than 0 03 units ml serum To obtain a maximum frequency of cure by means of a single injection therefore an increased dose of penicillin is recommended in the treatment of acute gonorrhoea

Minimum inhibitory concentration of penicillin was higher than 0 03 units ml serum in 16 1 per cent of all gonococcal strains isolated in this material and higher than 0 1 un ts ml serum in 7 8 per cent of the strains

57 male g norrhoea patients were also examined as to conc m tant presence of pen cillinase producing bacteria in the urethra Such bacteria were found in 33 of these cases When grouping the cases either according to concomitant presence of penicillinase producing bacteria frequ ency of recurrences or penicillin susceptibility of the g noc ccal strains isolated no statistically significant influence of penicilli ase producing bacteria in the urethra c uld be demonstrated on the results of penicillin therapy against gonorrhooea

Juhl n I & Krook G Institute of Clinical Bacteriology and Department of Dermat Venereol gy General H spital Malm RESULTS OF DIRECTED THERAPY IN GONORRHOEA To be published i Acta Derm Venere l Scand

Wallmark G Bacteriol gical Laborator S dersjukhuset Stockholm STUDIES OF FUCIDIN

The new ant biotic fucidin has a narrow antibacterial spectrum being active mainly on staphyl cocci All out of 208 strains of *Staph aureus* tested irrespective of their susceptibility to other available antibiotics were inhibited by 0 8 µg ml or less The same was true with the maj rity of 74 stra ns of gonoc cci tested Seven strains of g n cocci requiring m re than 0 8 µg ml to btain inhibiti n were also less sensitive to penicill n Other gram p sitive c cci and *H influenzae* requ red up to 100 µg ml to pr vide inhibiti n A variety of enterobacteria were resistant

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Meeting March 13, 1964, Stockholm Sweden

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Lodin A Nystrom B Rosengren S & Willen S Department of Dermatology and Department of Clinical Bacteriology Karolinska Sjukhuset Stockholm
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Jahl n I & Krook G Institute of Clinical Bacteriology and Department of Dermato Venere l gy General Hospital Malmo RESULTS OF DIRECTED THERAPY IN GONORRHOEA To be pul lished i Acta Derm Venereol Scand

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At transfers in dilution series of fucidin in broth highly resistant variants of staphylococci rapidly emerged The origin of these variants was shown to be a few cells in the population with significantly lower susceptibility Resistance did also develop in a few patients with serious staphylococcal infections given fucidin as the only drug This might have been prevented by combinations with another antibiotic

Good clinical response with fucidin was observed in cases of superficial staphylococcal infections like recurrent hidroadenitis and furuncles

Malmborg A S Department of Clinical Bacteriology, Karolinska Sjukhuset, Stockholm PATHOGENICITY TEST FOR STAPHYLOCOCCI WITH DNA PLATES

Staphylococcus aureus has been found to release large quantities of a calcium activated deoxyribonuclease (DNase), which other staphylococci do not produce (*Cunningham et al* J Am Chem Soc 78 4642, 1956) *Di Salvo* (Med Tech Bull 9, 191, 1958) reported a good correlation between DNase and coagulase activity

In the present experiments 110 Staphylococcus aureus strains and 65 Staphylococcus albus strains isolated from patients treated at Karolinska sjukhuset were tested for DNase as well as coagulase activity The DNase activity was determined as described by *Di Salvo* The strains were band streaked in sectors on a medium consisting of Trypticase Soy Agar with 2 mg DNA per ml (Baltimore Biological Laboratory), and incubated over night DNase activity was determined by flooding the plate with 1 M HCl Reaction of the acid with the nucleic acid in the medium yields a cloudy precipitate in all parts of the plate except those where DNA depolymerization has occurred

The correlation between the two methods was good Only one strain was DNase positive but coagulase negative One colony was sufficient to perform the test Apathogenic neisseria and enterococci the colonies of which resemble staphylococci were DNase negative The test could be used also in the presence of proteus when the clear zone of DNA depolymerization was seen under the swarming

Kjellander J & Myrback K F Department of Clinical Bacteriology Karolinska Sjukhuset Stockholm A SIMPLE TEST FOR PENICILLINASE PRODUCTION

The surface of a blood agar plate is seeded with a suspension of penicillin sensitive indicator organisms *i e* Staphylococcus aureus # 209 of Sarcina lutea A filter paper disc containing 20 IU of benzyl penicillin the same as used for the routine disc sensitivity test is placed in the centre of the agar surface The test organism is inoculated as a single radial streak from the disc to the edge of the agar surface and the plate is kept at room temperature for three hours to allow the penicillin to diffuse into the medium The test is read after overnight incubation at 37° C

If the test organism does not produce penicillinase the zone of inhibition of the indicator organism is circular If penicillinase is produced the indicator organism will grow along the sides of the streak as satellite colonies within the zone of inhibition

The test does not permit a differentiation of the different types of penicillinase nor does it give quantitative information It is however useful in the routine laboratory work

Nilsson L A & Moller, A J R., Department of Bacteriology, University of Gothenburg Gothenburg DETERMINATION OF BACTERIAL SENSITIVITY TO CHEMOTHERAPEUTIC AGENTS IN AEROBIC AND ANAEROBIC MILIEU

Sensitivity determination has been performed on facultative anaerobic bacterial strains against 16 chemotherapeutic agents and antibiotics (for the sake of convenience called chemotherapeutic agents) The strains tested were 35 coliforms 15 enterococci 26 alfa streptococci, and 22 gamma streptococci The disc method under standardized conditions with a specially *elaborated medium was used* The medium was based on Trypticase Soy Broth (BBL) with potato juice cysteine hydrochloride, glucose and defibrinated horse blood as additives This medium permits abundant growth of even fastidious microorganisms Determinations have been performed in duplicate, (2 plates incubated aerobically and 2 plates anaerobically) When the mean values *of the diameters of the* inhibition zones of the aerobically and anaerobically handled plates were compared, differences exceeding 4 mm were considered as significant

There was a definite tendency toward larger inhibition zones after aerobic incubation with sulphonamide streptomycin neomycin, methicillin, erythromycin oleandomycin and chloramphenicol while especially chlortetracycline gave larger zones after anaerobic incubation In regard to kanamycin penicillin tetracycline oxytetracycline, novobiocin polymyxin, bacitracin and nitrofurantoin some of the strains gave larger zones aerobically while others gave larger zones anaerobically

The cause of the difference in vitro might be due to an altered mode of action of the chemotherapeutic agent in the anaerobic as opposed to the aerobic metabolism *No doubt* there are *infectious conditions* where *one must influence facultative* anaerobic bacteria in the anaerobic metabolism If these findings can be applied *in vivo* these conditions might influence the results of antibiotic treatment

Wickman K Department of Clinical Bacteriology Karolinska Sjukhuset Stockholm SENSITIVITY TEST ON SEMISYNTHETIC MEDIA

Bacterial sensitivity tests according to the paper disc method performed on three different commercial semisynthetic media were compared to tests performed *in laboratory made horse blood* agar

The zone diameters of most of the antibiotics were found to differ rather much not only between blood agar an semisynthetic media but also between the different semisynthetic media In some instances the differences could be referred to the presence of components in the media which either partly inactivated or stimulated the activity of the antibiotic diffusing from he disc In others, *growth stimulating* or inhibiting factors in the media produced variations in the density of bacterial growth giving differences in the diameters. *For some antibiotics no such explana tion could be found*

A statistical analysis *of the difference between* blood agar and one of the semi synthetic media was made to determine a transformation factor allowing the tables constructed *for reading the inhibition* zones on blood agar to be used also for this medium

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TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting April 25, 1964

A. L. Obel, L. Nicander & A. Asheim: KIDNEY CHANGES IN DOGS WITH PYOMETRA

P. A. Persson, L. Nicander & L. Rutqvist: KIDNEY DAMAGE CAUSED BY ASPERGILLUS FUMIGATUS TOXIN

Oral administration to mice of endotoxin prepared from Aspergillus fumigatus by extraction of mycelial material with physiological saline caused no clinical signs or pathological changes. Paranteral administration caused, however, kidney damage. Twenty four hours after an intraperitoneal injection of 0.25 LD$_{50}$ for mice there was swelling of the glomerular epithelium, hyaline droplet degeneration and sometimes necrosis in the intermediary parts of the proximal convoluted tubules. After 48 hours there was widespread degeneration of the proximal convoluted tubules and necrosis of the intermediary segment. Electron microscopy showed alterations of many mitochondria and of the endoplasmic reticulum as well as numerous enlarged dense absorption droplets and often focal cytoplasmic degradation. In severely damaged tubules the epithelial hight was often less than 1 μ. After three and four days the glomerular changes persisted but the damaged tubules showed signs of repair.

N. E. Björklund & K. Erne: HISTO-PATHOLOGICAL STUDIES ON SPONTANEOUS ARYLPHOSPHATE INTOXICATION IN CATTLE

G. Lindblad: ULTRASTRUCTURAL AND INTRAVITAL MICROCIRCULATION STUDIES ON EXPERIMENTAL HEPATITIS CONTAGIOSA CANIS

A. Nilsson: ^{90}SR-INDUCED TUMOURS AND THE INFLUENCE OF PREGNANCY ON TUMOUR FREQUENCY AND LOCALISATION

S. Rubarth: THE CAUSE OF SUDDEN DEATH IN RACEHORSES

Acta path et microbiol scandinav 61 497 02 1964

The University Institute of Human Genetics (Head Professor Tage Kemp, M D)
Department of Experimental Genetics and Cytology (J Schultz Larsen M D),
Copenhagen Denmark

TWO CASES OF CHRONIC MYELOID LEUKAEMIA WITH PRESUMABLY IDENTICAL 47 CHROMOSOME CELL LINES IN THE BLOOD

By

BENT PEDERSEN

Received 14 x 63

The Philadelphia chromosome (Ph[1]), which was first described by *Nowell & Hungerford* in 1960 (1), has been found in mitoses in blood and/or bone marrow from almost all patients with chronic myeloid leukaemia examined till now In addition cell lines with chromosome abnormalities besides Ph[1] have been described in a number of patients (2, 3, 4 5, 6, 7, 8, 9, 10, 11) With one exception these abnormal cell lines have each been demonstrated in one patient only The exception is a 45-chromosome line lacking a small acrocentric chromosome, which has been found independently by three investigators (6, 10, 11)

In the present paper two patients with presumably identical 47 chromosome cell lines are described The lines are characterized by lacking a small, submetacentric chromosome and containing two supernumerary members of the middle group

CASE HISTORIES

Case 1 A 23 year old decorator who had been suffering for a year from abdominal distension and fatigue was hospitalized in June 1960 Physical examination revealed a pronounced splenomegaly The haemoglobin concentration was 9 1 g per cent the white blood cell count 114 000 per µl (myeloblasts 5 per cent promyelocytes 7 per cent myelocytes 16 per cent metamyelocytes 4 per cent polymorphonuclear neutrophils 47 per cent eosinophils 7 per cent basophils 10 per cent lymphocytes 3 per cent and normoblasts 1 per cent) The platelet count was 767 000 per µl Sternal puncture disclosed the presence of numerous immature cells of the myeloid series The patient had not been exposed to X rays except for diagnostic purposes Busulphan treatment using daily doses of 2 6 mg was started and continued until August 1962 At this time the splenomegaly increased the leucocyte number rose and Busulphan was replaced by 6 Mercaptopurine in daily doses of 150 mg combined with splenic irradiation (16 exposures of 10 15 r totalling 225 r) The clinical and haematological condition of the patient aggravated however and he died on August 31 1962 Times of blood sampling for chromosome examination together with relevant haematological data are presented in Table 1

Supported by a scholarship from the *Lady Tata Memorial Trust* and a grant from *Anders Hasselbalchs Fond* til Leukæmiens Bekæmpelse

TABLE 1

Times of Blood Sampling for Chromosome Examination together with Relevant Haematological Data

		Hgb	Pl	Leuc	Bl	PMy	My	MMy	N	Bd	Eos	Bas	Lymph	Mono	Nbl
Case A	18 7 62	12 3	270 000	84 000	6	4	8	–	53	5	2	14	8	–	–
	18 8 62	7 6	5 300	22 500											
	29 8 62	6 8	16 000	9 300	76	–	2	–	–	–	1	–	21	–	–
Case B	13 2 63	7 5	261 000	1 200	6	1	13	2	46	16	1	6	7	1	1
	12 3 63	10 7	375 000	18 400	13	3	11	3	39	11	2	11	4	1	2
	2 4 63	7 9	108 000	9 500	17	–	4	–	51	12	–	5	9	–	2
	30 4 63	4 8	129 000	50 000	35	3	20	3	20	4		8	3	–	4
	19 6 63	7 2	109 000	10 900	32	1	6		31	2	–	17	4	–	7

Hgb = haemoglobin (g per 100 ml) Pl = Platelets (per µl), Leuc = Leucocytes (per µl), Bl = Myeloblasts (per cent), PMy = Promyelocytes (per cent) My = Myelocytes (per cent), MMy = Metamyelocytes (per cent), N = Neutrophils, Bd = Bands (per cent), Eos = Eosinophils (per cent) Bas = Basophils (per cent), Lymph = Lymphocytes (per cent), Mono = Monocytes (per cent), Nbl = Normoblasts (per cent)

Case B A 21-year old student who for some weeks had felt abdominal distension and heaviness was examined in October 1959 and found to have splenomegaly The haemoglobin concentration was 86 per cent, the white cell count 187 000 per µl (mye- ... per cent myelocytes 18 per cent, metamye-

METHODS

leucocytes cultured in the pre- ... ification of the technique sug- ... s were stained with Giemsa All ... ication of the chromosomes ac-

RESULTS

Findings from the chromosome counts are presented in Table 2 In both cases mitoses with 47 chromosomes were more numerous at the later than at the first examinations Cells with 48 chromosomes or more were observed at the final three examinations of case B

TABLE 2

Results from Chromosome Counts

		<44	44	45	46	47	48	>48	+Ph¹	Ph¹?	−Ph¹	Total
Case A	18 7 62	–			6	7	–		13	–	–	13
	18 8 62	–		–	3	9	–	–	10	1	1	12
	29 8 62			1	8	36		–	42	2	1	45
Case B	13 2 62	–	1	4	42	3	–	–	2	3	45	50
	12 3 62		1	1	40	8	–		14	2	34	50
	2 4 63	2		2	15	28	2	1	44	–	6	50
	30 4 63				21	31	[illegible]	2	53	–	8	61
	19 6 63			–	10	15	–	8	30	1	2	33

+Ph¹ = cells with Ph¹, − Ph¹ = cells without Ph¹, Ph¹? = cells in which it has been impossible definitely to decide whether or not Ph¹ is present

In case A the presence of Ph¹ was demonstrated in more than 80 per cent of the examined cells Between March 12 and April 2, 1963 the Ph¹-incidence of case B *increased* steeply During this interval the clinical condition of the patient deteriorated significantly

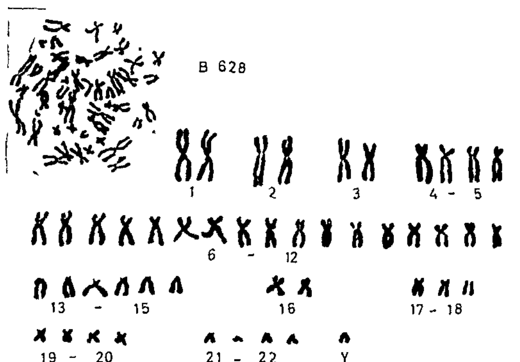


Fig 1

Karyotype with Ph¹, 47 chromosomes, two supernumerary members of the (6 X 12) group and lacking one member of the (17–18) group

In both cases chromosome analysis showed mitoses with 47 chromosomes in which a small, submetacentric member of the 17 18 group was lacking and two chromosomes of the 6 X 12 group were supernumerary, as illustrated in Fig 1 In case A the occurrence of this karyotype was seen to increase during the course of the disease In case B the demonstration of the karyotype coincided with a rise of the Ph¹-incidence and a declining haemoglobin concentration (Table 3)

TABLE 3

Incidence of Ph Positive Cells Belonging to the Abnormal Cell Lines in the individual Blood Samples

	Case A			Case B				
	18 7 1962	18 8 1962	29 8 1962	13 2 1963	12 3 1963	2 4 1963	30 4 1963	19 6 1963
Cells with Ph¹ (% of total cells)	100	91	98	4	29	88	87	94
Abnormal Strain (% of total cells)	23	33	67	0	0	46	48	42

In addition to the aforementioned chromosome abnormalities both patients presented a number of other abnormal karyotypes, although the numerical representation of these was lower Most of them were marked by the presence of supernumerary chromosomes

DISCUSSION

Previous studies have shown that the Ph^1 incidence decreases during therapeutically induced remissions (5, 11, 13) probably because Ph^1 positive cells are more sensitive to therapy e g cytotoxics than Ph^1 negative cells Case B showed the reverse development increasing incidence of Ph^1 positive cells during clinical relapse (Table 3) Both findings suggest a certain parallelity between (1) the clinical and haematological course and (2) changes of the Ph^1 incidence

It is obscure whether the cell lines of the two cases in fact are identical It is not possible with certainty to decide whether the lacking chromosome and the two supernumerary ones are the same in both patients

In case B demonstration of the abnormal cell line coincided with clinical and haematological relapse three and a half years after establishment of the diagnosis of chronic myeloid leukaemia The interval between the demonstration of the abnormal karyotype and the time of death covered less than six months In case A the same karyotype was demonstrated already at the first examination two and a half months before the death of this patient The time of its origin remains obscure

The abnormal karyotype is seen exclusively in cells with Ph^1 which apparently is conditional for its development Presumably each cell line has developed from a single ancestral cell by one or more mutations The increasing incidence of the cell lines in spite of cytotoxic treatment indicates a relatively low sensitivity to the applied drugs Such reduced sensitivity would constitute a significant selectional advantage to more sensitive cells If this hypothesis is correct the abnormal cell line probably plays an important part of the character and course of the disease as cells with the abnormal karyotype might be able to proliferate in spite of heavy treatment

SUMMARY

Chromosome analysis on cultured leucocytes from two young men with *chronic myeloid leukaemia* has demonstrated an abnormal Ph^1 positive cell line with 47 chromosomes in either case The karyotypes of both lines lacked a small submetacentric member of the 17 18 group and contained two supernumerary chromosomes of the 6 X 12 group The two patients were examined at three and five occasions respectively In one of the patients the abnormal cell line was found to have developed already at the first examination two and a half months prior to his death In the other patient the cell line developed during clinical and haematological relapse three and a half years after establishment of

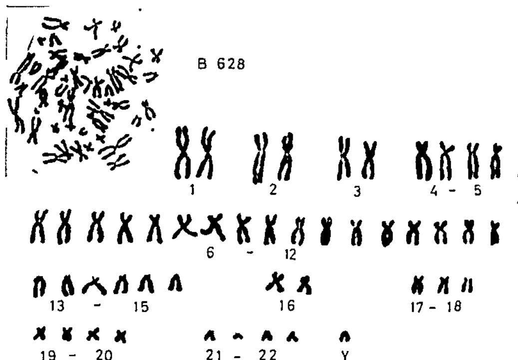


Fig 1

Karyotype with Ph¹, 47 chromosomes, two supernumerary members of the (6-X-12) group, and lacking one member of the (17 18) group

In both cases chromosome analysis showed mitoses with 47 chromosomes in which a small, submetacentric member of the 17–18 group was lacking and two chromosomes of the 6–X–12 group were supernumerary, as illustrated in Fig 1 In case A the occurrence of this karyotype was seen to increase during the course of the disease In case B the demonstration of the karyotype coincided with a rise of the Ph¹-incidence and a declining haemoglobin concentration (Table 3)

TABLE 3

Incidence of Ph Positive Cells Belonging to the Abnormal Cell Lines in the individual Blood Samples

	Case A			Case B				
	18 7 1962	18 8 1962	29 8 1962	13 2 1963	12 3 1963	2 4 1963	30 4 1963	13 6 1963
Cells with Ph¹ (% of total cells)	100	91	98	4	29	88	87	94
Abnormal Strain (% of total cells)	23	33	67	0	0	46	48	42

In addition to the aforementioned chromosome abnormalities both patients presented a number of other abnormal karyotypes, although the numerical representation of these was lower Most of them were marked by the presence of supernumerary chromosomes

DISCUSSION

Previous studies have shown that the Ph¹-incidence decreases during therapeutically induced remissions (5, 11, 13), probably because Ph¹-positive cells are more sensitive to therapy, e g cytotoxics, than Ph¹-negative cells Case B showed the reverse development increasing incidence of Ph¹ positive cells during clinical relapse (Table 3) Both findings suggest a certain parallelity between (1) the clinical and haematological course and (2) changes of the Ph¹-incidence

It is obscure whether the cell lines of the two cases in fact are identical It is not possible with certainty to decide whether the lacking chromosome and the two supernumerary ones are the same in both patients

In case B demonstration of the abnormal cell line coincided with clinical and haematological relapse three and a half years after establishment of the diagnosis of chronic myeloid leukaemia The interval between the demonstration of the abnormal karyotype and the time of death covered less than six months In case A the same karyotype was demonstrated already at the first examination two and a half months before the death of this patient The time of its origin remains obscure

The abnormal karyotype is seen exclusively in cells with Ph¹, which apparently is conditional for its development Presumably each cell line has developed from a single ancestral cell by one or more mutations The increasing incidence of the cell lines in spite of cytotoxic treatment indicates a relatively low sensitivity to the applied drugs Such reduced sensitivity would constitute a significant selectional advantage to more sensitive cells If this hypothesis is correct, the abnormal cell line probably plays an important part of the character and course of the disease, as cells with the abnormal karyotype might be able to proliferate in spite of heavy treatment

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Chromosome analysis on cultured leucocytes from two young men with chronic myeloid leukaemia has demonstrated an abnormal Ph¹-positive cell line with 47 chromosomes in either case The karyotypes of both lines lacked a small, submetacentric member of the 17 18 group and contained two supernumerary chromosomes of the 6–X–12 group The two patients were examined at three and five occasions, respectively In one of the patients the abnormal cell line was found to have developed already at the first examination two and a half months prior to his death In the other patient the cell line developed during clinical and haematological relapse three and a half years after establishment of

the diagnosis of chronic myeloid leukaemia and six months prior to his death Immediately after the demonstration of the abnormal cell-line of the latter patient, the Ph[1]-incidence was seen to rise steeply The increasing incidence of the line in both patients in spite of cytotoxic treatment suggests a reduced sensitivity to the drugs applied In that case the abnormal cells may be able to proliferate in spite of heavy treatment and thus be responsible for the deterioration of the clinical condition of the patients

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ACCIDENTAL CHLOROFORM NEPHROSIS IN MICE

By

L Jacobsen, E Krag Andersen, J V Thorborg

Received 19 xi 63

This study discusses a disease affecting the kidneys—"chloroform nephrosis"—seen in male mice accidentally exposed to chloroform The disease may not be generally known and although it has been described before, it may be of interest for laboratory workers engaged in experimental work to have their attention directed to the condition because the rate of mortality among mice exposed to even very small concentrations of chloroform in the air may be quite high

Survey of Literature

Reports are available concerning the development of nephrosis following accidental or experimental exposure to chloroform in which the authors ascribe to chloroform the patho anatomical renal lesions characteristic of nephrosis *Hewitt*, demonstrated in 1956 that even minute quantities of chloroform vapours in the atmosphere were sufficient to provoke renal changes After a period during which the laboratory staff were on holiday and the ventilation in the animal rooms may thus have been somewhat unsatisfactory, it was observed accidentally that numerous cases of a uniform, not infrequently fatal, disease occurred among the mice It was not possible to demonstrate the presence of toxic agents, and it was therefore concluded that, as glass stoppered containers with chloroform were kept in the rooms in question, pollution of the air by chloroform might have been responsible for the state of affairs

Subsequently that author was successful in reproducing the disease and using various fixed doses of chloroform, it was demonstrated that even a very low concentration of chloroform in the air (1 2 mg or less per litre for two hours) was sufficient to cause the development of renal lesions including widespread tubular necrosis within 24 hours In

The authors wish to thank Dr *Mogens Hauge* M D for most valuable discussions during the work

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agreement with the findings in the present study, Hewitt observed that only male mice were affected, while the young offspring showed no signs of disease. That author also demonstrated that it is possible to provoke chloroform nephrosis in female mice treated previously with androgenous hormones.

The patho-anatomical picture of chloroform nephrosis was first described by *Eschenbrenner* (1945). In a later report (*Eschenbrenner & Miller* 1945) it was declared impossible to produce similar changes in female mice and experiments using castrated mice were equally negative. If, however, the latter were treated with androgenous hormones they presented the typical picture of "extensive necrosis of portions of the proximal and distal, convoluted tubes", but no changes were observed at the site of the Bowman capsule and the first portion of the proximal convoluted tube. In the same study, attention was directed for the first time to the different histological composition of the Bowman capsule in male and female mice. In the latter, the capsule is enclosed by a very flat epithelium, while in the former it is cuboidal and distinguishable only with difficulty from the epithelium in the proximate convoluted tubules. It was observed—also for the first time—that chloroform may provoke necrosis of the liver.

Shubik & Ritchie (1953) found the same characteristic renal changes in male mice accidentally exposed to very low concentrations of chloroform in the air, the female mice were unaffected.

Deringer, Dunn & Heston (1953) observed 100 per cent mortality of male C3H mice after exposure to 5 mg of chloroform per litre air for 1, 2 and 3 hours. The first deaths occurred within a couple of days and the last after intervals of 14 to 18 months. In the male mice which died within a few days after exposure, the characteristic renal changes were found to be combined with necrosis of the liver. That feature was not seen in animals which survived for longer periods. In addition, the resistance of the various inbred strains was found to be different. The authors attributed that difference to a genetic factor.

Description of the Disease

Both in the study by *Hewitt* and in the present work, a number of causal observations led to the performance of experiments with a view to examining further the chloroform nephrosis. In the course of immunization experiment using B. pertussis carried out at Statens Seruminstitut in the years 1953–54 (*Krag Andersen et al.* 1958) and in radiation experiments carried out at the University Institute for Human Genetics (*Jacobsen* 1961), sometimes about 50 per cent of the experimental mice became ill and presented characteristic renal lesions on death. In the most severe cases, the mice became ill within 3 to 4 days. Generally the animals would be found lying prone with every hair erect and seized by violent tremors. The animals died within 10 to 12 days.

Only in the C3H mice was death seen to occur within 1 to 2 days In less [illegible] symptoms were minimal and the disease [illegible]

recovered within 6 to 7 days

Own Investigations

This characteristic disease was observed in four different strains of mice and also in wild house mice Parallel experiments carried out in male and female mice showed beyond any doubt that only male mice were affected, and chiefly those older than 6–7 weeks The disease was particularly conspicuous in mice vaccinated with pertussis vaccine and subsequently exposed to intracerebral or intranasal infection with B pertussis However, the disease could also develop in untreated mice

All attempts to demonstrate the presence of a bacterial or viral infection were negative To a certain degree the histological picture resembled that seen in man after corrosive sublimate poisoning, thus indicating a toxic action Consequently mice were exposed to the action of almost all kinds of material with which they might have been in contact in the laboratory The results of these experiments were also negative

The disease occurred at a time when intranasal infection with B pertussis had been introduced parallel with experimental, intracerebral infection The latter technique requires that mice are anaesthetized with ether as opposed to the former where a mixture of ether and chloroform is used This brought chloroform into focus as a conceivable causative agent in the poisoning, but as the disease occurred in mice which had not been anaesthetized, and also in mice anaesthetized with ether or a mixture of ether and chloroform, it was assumed that this factor could be ruled out

As only male mice were affected by the renal disease, the experimental work with B pertussis was continued using only female mice The origin of the renal lesions remained unexplained until 1956 when *Hewitt* described a disease in mice which apparently was identical, both clinically and patho anatomically, with the disease observed in the present experiments

Subsequently it was possible to confirm that chloroform could produce a pathological picture which closely resembles that observed by the authors in 1953 54, although it is not considered certain that the development of the disease is attributable exclusively to chloroform poisoning

Several experiments in which graduated cylinders containing chloroform were kept in the animal rooms gave very varied results The typical pathological picture was encountered in all the experiments, but the number of mice affected fluctuated from a few per cent up to more

TABLE 1

The Sensitivity of Mice to Chloroform Diluted in Groundnut Oil and Administered by Stomach Pump

Mouse strain	USA		C3H				C									
Sex Weight (g)	♂ 20 24		♂ 20–24		30–38		♀ 20 24		♂ 20 24							
	Non treated										Hormone treatment*		Castrated		Sham operated	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Exp 1	178 (54)	100	250 (24)	100					178 (60)	90						
Exp 2	115 (21)	100			79 (15)	45			89 (21)	75						
Exp 3							<40 (15)	0	<40 (15)	92	79 (15)	71	40 (18)	18	224 (21)	90
Exp 4															250 (18)	°
Exp 5															51§ (30)	°
Exp 5															36† (30)	°

a) LD 50 expressed as the reciprocal dilution factor Volume 0 5 ml/ mouse
b) Percentage of mice showing specific kidney changes

Figures in brackets Total number of mice divided into 3 groups each given chloroform in dilutions 1 40, 1 100, and 1 250
* Oestradiol benzoate 50 µg/ml 0 2 ml subcutaneously Three doses with 3 day intervals starting the day before feeding by stomach pump
§ The mice were subjected to superficial operation not involving the testicles
† The mice were subjected to a less superficial operation not injuring the testicles
° Not investigated

than 70 per cent The intensity of the disease was also seen to differ. Modification of the experimental conditions, e g. varying times of exposure to chloroform vapours, anaesthesia using either ether or chloroform or a mixture of both, the use of mice either unvaccinated or immunized against pertussis and subsequently challenged intranasally or intracerebrally with B pertussis, were found to be without importance as regards the intensity of the disease.

If mice were fed by stomach pump with chloroform diluted in groundnut oil, an excellent correlation between mortality and dosage was found The pathological picture of the kidneys was characteristic, particularly in the mice which died a few days after administration and in those sacrificed at later stages Not all the mice showed renal lesions

Table 1 shows the fatal action of chloroform on mice of three different strains Peroral chloroform in groundnut oil was given by stomach pump in doses of 0 5 ml, the dilutions being as follows. 1. 40, 1: 100 and 1 250 The sensitivity of the mice is expressed by the degree of dilution capable of killing 50 per cent of the animals within 10 days (LD 50) The surviving mice were sacrificed on the tenth day following administration Histological examination of the kidneys was carried out in all cases The figures column b indicate in percentage the renal changes recorded in the individual groups The degree of the lesions varied from weak to highly typical In addition, the table records the results of chloroform poisoning in castrated, hormone-treated and "sham-operated" male C mice exposed to an operation which did not injure the testicles These mice served as controls for the castrated mice

Three experiments carried out with non treated male C mice show that the LD 50 ranges between <40 and 178, a variation which is too marked to permit demonstration of differences in the sensitivity of the individual strains

In two experiments the "sham-operated" mice were found to be highly sensitive to chloroform poisoning, but the finding could not be verified in the third experiment

Attempts to explain why the results were unsuccessful are futile, but it can hardly be doubted that factors other than chloroform may have contributed to producing the typical renal lesions

Table 2 records the number of deaths which occurred in each group

TABLE 2

Mortality Rate in Two Strains of Mice Affected with Nephrosis

Strain	H				C3H	
	Irradiated group		Breeding stock			
Total	200 ♂	200 ♀	120 ♂	300 ♀	75 ♂	100 ♀
Number of deaths	26 ♂	1 ♀	24 ♂	2 ♀	50 ♂	0 ♀

in the course of radiation experiments carried out at the University Institute for Human Genetics In the H strain mice, deaths occurred sporadically within about one month, as opposed to the C3H mice in which deaths occurred within a few days The ages of the dead animals ranged between 130 and 346 days It should be borne in mind that no infectious diseases had been observed among the animals during the last five years, and for the last three years no fresh animal strains had been introduced The mice were given food and water ad libitum

With a view to obtaining a rough estimate of the importance of genetic factors, if any, an experiment was carried out based on the findings in male strain H mice which had died from this type of nephrosis at times when the disease was fulminant In addition, the series included a control group of male, surviving mice which at the time of illness had been of almost the same ages as those which died

A study of paternal ancestors (parent, grandparent and great-grandparent) showed that nephrotic and healthy animals need not originate from two different groups of ancestors, but rather that the ancestors of sick and healthy animals had produced offspring in which the distribution affection/non-affection was found to be identical In such highly inbred strains (pure brother/sister breeding for 60 generations) it is hardly surprising that, to a great extent, the ancestors in the two groups were the same

This provides no basis for presuming that deaths in the H strain mice were attributable to a certain depression due to inbreeding

Following the removal of chloroform from the animal rooms, no deaths of this nature have been seen during a period of more than 12 months The mortality rate has normalized, the numbers of deaths being distributed evenly in all groups

OWN INVESTIGATIONS

Pathological Anatomy

Gross inspection In the least severe cases the patho anatomical findings in dead animals from the two laboratories included well filled and hyperaemic kidneys with slightly granulated surfaces In the more severe cases the parenchyma was enlarged its colour being a little paler than normal In the most severe cases the kidneys were markedly enlarged the weights ranging from 50 to 75 per cent above normal The surface was delicately granulated and the colour pale to greyish white On

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Figs 1 4

Fig 1 Synoptic microphoto of the kidney from a mouse which died spontaneously note the marked calcareous precipitation in the cortex Magnification × 8

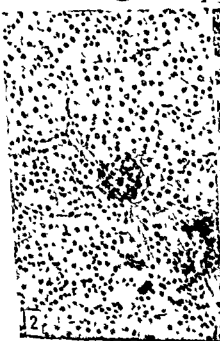
Fig 2 Microphoto of renal cortex from a normal male mouse Magnification × 300

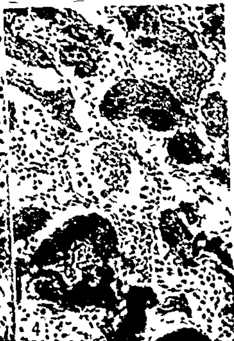
Fig 3 Microphoto of renal cortex from an affected male mouse Magnification × 150

Fig 4 Same kidney as in Fig 3 Magnification × 300 Note the well preserved epithelium in the Bowman capsule and in the proximal sections of the first convoluted tubule The dark granulated mass is solid calcareous precipitations in the necrotic epithelium of the tubule


1


3


2


4

section the cortex was found to have widened, presenting finely granulated, calcareous deposits clearly distinguishable by cutting A diagram af a severely affected kidney showing calcareous, not quite

Histological examination Initial cha

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the cortical convoluted tubules The lumina were dilated and filled with an eosinophile, proteinic material and cellular desquamation In fulminant, acute cases in which death occurred within a few days, the tubular epithelium could be completely *eliminated by* necrosis, the remaining cells being discernible as a structureless zone on the basal membrane In more protracted cases hydropic degeneration of the cellular protoplasms was seen Particularly characteristic was the precipitation of numerous, markedly haematoxylin stained granules of various sizes in the swollen *protoplasma* These might coalesce into a calcareous zone forming the line of demarcation of the subules (Fig 3) In severe, protracted cases even gross inspection disclosed the well defined borderline separating the severely affected cortex from *the much less affected* central portion of the kidney The glomeruli were not primarily affected and the cuboid epithelium, seen in male mice on the internal side of the Bowman capsule (*Eschenbrenner* 1945), together with the most proximal portion of the first convoluted tubule, remained quite unaffected Prior to the occurrence of the calcareous precipitation the picture might resemble the bilateral cortical renal necrosis known from human pathology The calcareous precipitations might give it a certain resemblance to the necrosis attributable to corrosive sublimate poisoning

All the lesions described were seen both in animals "spontaneously" affected with the disease as well as in animals experimentally exposed to chloroform The most susceptible animals died within one or two days, and presented total "coagulation necrosis" of the tubular epithelium, but the calcareous precipitations were not seen, except in animals which survived for 10 days or more

Not all the spontaneously affected animals died, and examination of the kidneys from 33 animals which had survived feeding by stomach pump of various doses of chloroform showed the typical changes in 10 of the cases

DISCUSSION

From a patho anatomical point of view, there can scarcely be any doubt that the renal lesions observed in the present study are identical with the changes reported in the literature Both at Statens Seruminstitut and at the University Institute for Human Genetics, it has been a case of exposure to rather low chloroform concentrations However, these were as high or even higher than the concentration used by *Hewitt* Moreover, the same patho-anatomical changes were noted in our experiments, including exposure to fixed though rather high concentrations These findings point to chloroform as the exogenous causative agent in the disease, which develops under highly divergent experimental conditions The finding is further substantiated by our vain attempts to demonstrate any action of other exogenous factors Finally the renal changes seen in one of the strains (strain H) were assumed to be interpretable as the result of a depression due to the high degree of inbreeding However, further examinations failed to substantiate that assumption

Hence, it is reasonable to assume that the renal necrosis observed in

our laboratories must be interpreted—in full or in part—as the result of some accidental exposure to chloroform Our investigations also seem to confirm the observations concerning chloroform nephrosis in mice reported previously by *Hewitt*

Chloroform may also provoke necrosis of the liver (*Eschenbrenner & Miller* 1945) Only the renal nephrosis has been discussed in the present study but it should be added that marked necrosis of the liver was observed in several cases in which the course was typical

Although it must be admitted that chloroform is responsible for the nephrosis which developed in laboratory mice, it is still considered open to discussion whether the nephrosis is provoked by a direct action of chloroform on the kidneys or whether it may be a matter of some indirect action in which one or more other factors are responsible Various features seem to point to the latter possibility as the most probable

As regards the resistance to the disease the peculiar sex difference seen both in the present work and in most of the other studies should be emphasized Were this a question of a mechanism the action of which was attributable to chloroform exclusively the incidence would have been identical in male and female mice However this was not the case The androgenous or oestrogenous hormone levels might be assumed to interfere with this mechanism in some way or other Here attention should be directed also to the different anatomical composition of the Bowman capsule in male and female mice (*Eschenbrenner & Miller* 1945) There is conflicting evidence whether or not this is of any importance

One more factor should be mentioned viz the age variation *Hewitt* found that only adult males were susceptible though to varying degrees He found no effect in female and young animals and that observation is substantiated by the present findings Such age variations as regards *morbidity should* be considered probably on the basis of a possible hormonal influence on the pathogenesis of the syndrome

Thirdly in comprehensive materials some of the inbred strains were found to be sensitive and others resistant to the disease (*Deringer, Dunn & Heston* 1953) Even though the rather rudimentary genetic examination performed by us failed to reveal any genetic origin it is difficult bearing in mind the findings of *Deringer et al* to preclude the presence of a genetic factor in the action mechanism

In our laboratories the syndrome has been of quite epidemic character with outbreaks *occurring and* waning again not infrequently at intervals of several years despite the fact that the use and storage of chloroform has continued without interruption At the University Institute for Human Genetics the disease has occurred on two occasions in a period of four years Male mice which died showed the characteristic picture of chloroform necrosis including the renal lesions Thus it is rather surprising that an almost permanent exposure of male and

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section the cortex was found to have widened, presenting finely granulated, calca reous deposits clearly distinguishable by cutting A diagram af a severely affected kidney showing calcareous, not quite fresh, deposits is seen in Fig 1

Histological examination Initial changes were manifest in the form of hyperaemia in both medulla and cortex, followed by progressive necrosis of the epithelium of the cortical convoluted tubules The lumina were dilated and filled with an eosinophile, proteinic material and cellular desquamation In fulminant, acute cases in which death occurred within a few days, the tubular epithelium could be completely eliminated by necrosis, the remaining cells being discernible as a structureless zone on the basal membrane In more protracted cases hydropic degeneration of the cellular protoplasms was seen Particularly characteristic was the precipitation of numerous, markedly haematoxylin stained granules of various sizes in the swollen protoplasma These might coalesce into a calcareous zone forming the line of demarcation of the subules (Fig 3) In severe, protracted cases even gross inspection disclosed the well defined borderline separating the severely affected cortex from the much less affected central portion of the kidney The glomeruli were not primarily affected and the cuboid epithelium, seen in male mice on the internal side of the Bowman capsule (*Eschenbrenner* 1945), together with the most proximal portion of the first convoluted tubule, remained quite unaffected Prior to the occurrence of the calcareous precipitation the picture might resemble the bilateral cortical renal necrosis known from human pathology The calcareous precipitations might give it a certain resemblance to the necrosis attributable to corrosive sublimate poisoning

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DISCUSSION

From a patho-anatomical point of view, there can scarcely be any doubt that the renal lesions observed in the present study are identical with the changes reported in the literature Both at Statens Seruminstitut and at the University Institute for Human Genetics, it has been a case of exposure to rather low chloroform concentrations However, these were as high or even higher than the concentration used by *Hewitt* Moreover, the same patho-anatomical changes were noted in our experiments, including exposure to fixed, though rather high concentrations These findings point to chloroform as the exogenous causative agent in the disease, which develops under highly divergent experimental conditions The finding is further substantiated by our vain attempts to demonstrate any action of other exogenous factors Finally, the renal changes seen in one of the strains (strain H) were assumed to be interpretable as the result of a depression due to the high degree of inbreeding However, further examinations failed to substantiate that assumption.

Hence, it is reasonable to assume that the renal necrosis observed in

our laboratories must be interpreted—in full or in part—as the result of some accidental exposure to chloroform. Our investigations also seem to confirm the observations concerning chloroform nephrosis in mice reported previously by Hewitt.

Chloroform may also provoke necrosis of the liver (*Eschenbrenner & Miller* 1945). Only the renal nephrosis has been discussed in the present study, but it should be added that marked necrosis of the liver was observed in several cases in which the course was typical.

Although it must be admitted that chloroform is responsible for the nephrosis which developed in laboratory mice, it is still considered open to discussion whether the nephrosis is provoked by a direct action of chloroform on the kidneys, or whether it may be a matter of some indirect action in which one or more other factors are responsible. Various features seem to point to the latter possibility as the most probable.

As regards the resistance to the disease, the peculiar sex difference seen both in the present work and in most of the other studies should be emphasized. Were this a question of a mechanism, the action of which was attributable to chloroform exclusively, the incidence would have been identical in male and female mice. However, this was not the case. The androgenous or oestrogenous hormone levels might be assumed to interfere with this mechanism in some way or other. Here attention should be directed also to the different anatomical composition of the Bowman capsule in male and female mice (*Eschenbrenner & Miller* 1945). There is conflicting evidence whether or not this is of any importance.

One more factor should be mentioned, viz. the age variation. *Hewitt* found that only adult males were susceptible, though to varying degrees. He found no effect in female and young animals, and that observation is substantiated by the present findings. Such age variations as regards morbidity should be considered probably on the basis of a possible hormonal influence on the pathogenesis of the syndrome.

Thirdly, in comprehensive materials some of the inbred strains were found to be sensitive and others resistant to the disease (*Deringer, Dunn & Heston* 1953). Even though the rather rudimentary genetic examination performed by us failed to reveal any genetic origin, it is difficult bearing in mind the findings of *Deringer et al.*, to preclude the presence of a genetic factor in the action mechanism.

In our laboratories the syndrome has been of quite epidemic character with outbreaks occurring and waning again, not infrequently at intervals of several years, despite the fact that the use and storage of chloroform has continued without interruption. At the University Institute for Human Genetics the disease has occurred on two occasions in a period of four years. Male mice which died showed the characteristic picture of chloroform necrosis, including the renal lesions. Thus it is rather surprising that an almost permanent exposure of male and

female mice in all age-groups for several years resulted in two outbreaks only, both of which were of brief duration and occurred after an interval of 18 months. We are inclined to presume that the reason should be traced to one or more of the above-mentioned hypothetical conditioning factors

CONCLUSION

It must be concluded that the pathological picture manifest in our strains of mice in the form of patho-anatomical lesions of the kidney and liver, is attributable to aspiration of minute concentrations of chloroform. The action mechanism of chloroform is obscure, but it is difficult to preclude some interplay of other factors as well, probably of a hormonal or genetic character

Laboratory workers engaged in experimental work with mice are advised not to use chloroform in animal rooms, either for killing or for anaesthesia, and to refrain from storing chloroform in such animal quarters, irrespective of whether or not the stoppers of the containers are adequate It has been proved experimentally that in one strain of mice exposure to low concentrations of chloroform (less than 2 mg per litre of air for two hours) may provoke or contribute to the development of renal lesions, the outcome of which may be fatal

SUMMARY

The course of chloroform nephrosis and its patho-anatomical picture in mice are discussed on the basis of accidental exposure to chloroform of various strains of mice in two laboratories An evaluation of the renal changes observed in our cases, together with a comparison with experimentally provoked cases reported in the literature, shows reasonable accordance On the basis of studies of the literature, combined with our own experimental results, the aetiology and pathogenesis are discussed It is concluded that the syndrome is ascribable to chloroform, but the further mechanism of the action of this substance remains obscure Hormonal and genetic factors may be involved Work with and storage of chloroform should not take place in animal rooms

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The Department of Biochemistry, Statens Seruminstitut, Copenhagen, Denmark

DEMONSTRATION OF ERYTHROPOIETIN IN URINE AND IN KIDNEY EXTRACTS FROM RABBITS WITH EXPERIMENTAL CONSTRICTION OF THE LEFT RENAL ARTERY

By

POUL HANSEN

Received 18 xii 63

Since Jacobson demonstrated that erythropoiesis could not be stimulated by the administration of cobalt to bilaterally nephrectomized animals (*Jacobson, Goldwasser, Fried & Plzak* 1957) attention has to an increasing extent been turned to the problem of the ability of the kidney to produce the humoral factor erythropoietin It has thus been demonstrated that the number of reticulocytes increased in the peripheral blood of rats after one kidney had been twisted around the renal pedicle (*Mantz, Cholevas & Warter* 1960) *Nakao, Takaku, Tashiro, Tanaka, Oka & Imamura* (1960) have demonstrated an increase in the erythropoietin in the blood of rabbits after the application of a Goldblatt clamp to one of the renal arteries

Methods of concentrating erythropoietin from kidney tissue have previously been worked out in this laboratory (*Hansen* 1963 b) It has thus been made possible to search for more specific evidence that a kidney whose artery is partially occluded produces erythropoietin, by comparing the erythropoietin content of such a kidney with that of the contralateral kidney from the same animal

METHODS

Constriction of the left renal artery in rabbits has been carried out by means of silver clamps of the same type as those used by *Pickering & Prinzmetal* (1938) In order to avoid blood loss during the operation the left renal artery was approached through the peritoneum after an incision precisely along the linea alba The operation has been described previously (*Hansen* 1964)

Four groups of rabbits were studied Group 1 comprised seven animals Owing to deaths only four sets of kidneys were available for study The interval between operation and the killing of the animals was 37 days

Amongst the next 32 rabbits to undergo the operation there were no deaths in connection with the operation or during the first two postoperative days Of these rabbits 17 were included in group 2 Three of the rabbits in this group died spontane

The author expresses his thanks to Mr *B Mansa* (Pharmacist Dept of Biophysics) for carrying out and describing the immuno electrophoretic investigations

ously, while the remainder were killed because of weight loss The average life span

... days ... days before

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cardiac puncture

At section all the clamps in the rabbits in groups 2 and 3 were found to be in position They were embedded in fibrous tissue Immediately after removal the kidneys were cooled to —18° C and stored at this temperature until they were required for use

Blind Operation

Five rabbits (group 4) underwent the same operation The only difference was that the arterial clamps were removed immediately, during the operation

Production of the Kidney Preparations

tated with ethanol Details of the process have been published previously ... 1963 b)

Demonstration of Erythropoietin

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in all tests after test no 55

The haemoglobin concentration of the test rats was checked to ensure that positive results were not of a non specific nature Five rats were used for each test

Preparation of Urine

the ammonium acetate ethanol process (*Hansen* 1963 c)

Immuno electrophoresis was carried out using Scheidegger's method as modified by *Lind Mansa & Olesen* (1963) Antisera from goats immunized with rabbit serum were used

RESULTS

Estimation of Erythropoietin in Urine

The results are shown in Table 1 On erythropoietin testing the pooled urine from the ... ...gical response ... control value

TABLE 1

Estimation of Erythropoietin in Urine of Rabbits which Had Undergone the Goldblatt Operation

Test no	Group of rabbits	Clamp	Conc	Biological response % Fe^{59} incorp	Mean interval between operation and urine collection in days
A 45	1	+	8 ×	21.3 ± 4.3	8
C 56	2	—	8 ×	3.7 ± 0.5	b.o.
B 56	2	+	8 ×	12.4 ± 3.7	9
D 59	3	—	8 ×	5.0 ± 1.0	b.o.
C 59	3	+	8 ×	10.9 ± 1.4	5
F 61	3	+	4 ×	10.4 ± 2.1	51
D 64	4	—	8 ×	3.5 ± 2.0	8

Urine was collected before (b.o.) or after operation from the various groups of rabbits. The material used in test F 61 was obtained exclusively from the polycythaemic rabbit no. 9468. The rabbits in group 4 underwent blind operation. The protein content of the urine was concentrated by means of the tannic acid method before the erythropoietin test. For further details see text.

Urine was collected from some of the rabbits in groups 2 and 3 both before and after operation. It was possible to demonstrate the presence of erythropoietin in the sample of the pooled urines collected after operation, but not in the urine collected from the same animals before operation.

In urine collected exclusively from rabbit no. 9468 at a time when it had pronounced polycythaemia, erythropoietin could be demonstrated with certainty.

Urine collected from the rabbits subjected to blind-operation did not contain demonstrable amounts of erythropoietin.

None of the rabbits presented macroscopic haematuria.

TABLE 2

Erythropoietin Determination of Kidney Extracts

Group of rabbits	Number of rabbits	Days after operation	Clamp	Test no	Biological response % Fe^{59} incorp	g kidney per test rat
1	4	37	—	A 47	5.6 ± 0.3	4
			+	B 47	6.3 ± 1.9	4
2	18	9.6 (mean)		F 55	10.6 ± 3.8	16
			+	L 55	19.1 ± 2.0	16
3	15	>35	—	B 64	3.1 ± 1.8	16
			+	C 64	15.8 ± 2.8	16

An arterial constriction was produced by a silver clamp applied to the left renal artery in rabbits.

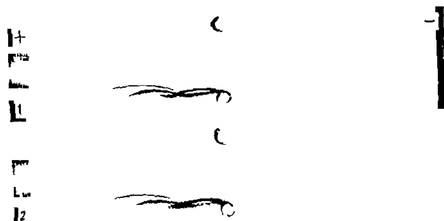


Fig 1
Immuno electrophoretic investigation of preparations of the pooled clamped kidneys (1) and the contralateral kidneys (2)

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Demonstration of Erythropoietin in Kidney Preparations

Table 2 shows that erythropoietin was not demonstrated in the preparations of the clamped kidneys from group 1 The results show that there was the same biological response to physiological saline as to extracts of both left and right kidneys

The extracts prepared from the clamped kidneys in groups 2 and 3 definitely contained more erythropoietin than did the extracts of the contralateral kidneys

Immuno Electrophoresis of the Kidney Preparations

After the estimation of erythropoietin the protein content of the remainder of the preparation produced from the kidneys of animals in group 3 was dialysed and concentrated by freeze drying

Two antisera from goats immunized with rabbit serum were used in the immuno electrophoresis Fig 1 shows that it was possible to demonstrate two *a* globulins in preparations of the right as well as of the left kidneys Normal rabbit serum has been used for comparison at the base of the plate Immuno electrophoretic investigation using the other anti serum revealed that the kidney preparations also contained small amounts of protein compounds which had the same mobility as albumin

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We have attempted to demonstrate the presence of erythropoietin in the heparin plasma obtained at section of some of the rabbits in

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C 59	3	+	8 ×	10.9 ± 1.4	5
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In urine collected exclusively from rabbit no 9468 at a time when it had pronounced polycythaemia, erythropoietin could be demonstrated with certainty

Urine collected from the rabbits subjected to blind-operation did not contain demonstrable amounts of erythropoietin

None of the rabbits presented macroscopic haematuria

TABLE 2

Erythropoietin Determination of Kidney Extracts

Group of rabbits	Number of rabbits	Days after *operation*	Clamp	Test no	Biological response *% Fe^{59} incorp*	g kidney per test rat
1	4	37	–	A 47	5.6 ± 0.3	4
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2	18	9.6 (mean)	—	E 55	10.6 ± 3.8	16
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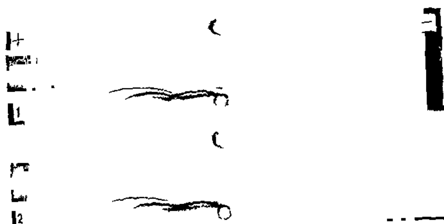


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Immuno-Electrophoresis of the Kidney Preparations

After the estimation of erythropoietin the protein content of the remainder of the preparation produced from the kidneys of animals in group 3 was dialysed and concentrated by freeze-drying

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B 56	2	+	8 ×	12 4 ± 3 7	9
D 59	3	—	8 ×	5 0 ± 1 0	b o
C 59	3	+	8 ×	10 9 ± 1 4	5
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None of the rabbits presented macroscopic haematuria

TABLE 2

Erythropoietin Determination of Kidney Extracts

Group of rabbits	Number of rabbits	Days after operation	Clamp	Test no	Biological response % Fe^{59} incorp	g kidney per test rat
1	4	37	—	A 47	5 6 ± 0 3	4
			+	B 47	6 3 ± 1 9	4
2	18	9 6 (mean)	—	E 55	10 6 ± 3 8	16
			+	F 55	19 1 ± 2 0	16
3	15	>35		B 64	3 1 ± 1 8	16
			+	C 64	15 8 ± 2 8	16

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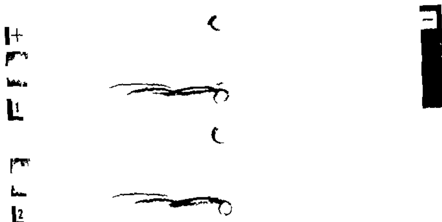


Fig 1

ncrease the protein content The degree of concentration in 1 and 2 [illegible]
At the bottom of the plate normal rabbit serum is used
The antisera used were from a goat immunized with rabbit serum

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C 56	2	—	8 ×	3 7 ± 0 5	b o
B 56	2	+	8 ×	12 4 ± 3 7	9
D 59	3	—	8 ×	5 0 ± 1 0	b o
C 59	3	+	8 ×	10 9 ± 1 4	5
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None of the rabbits presented macroscopic haematuria

TABLE 2

Erythropoietin Determination of Kidney Extracts

Group of rabbits	Number of rabbits	Days after operation	Clamp	Test no	Biological response % Fe⁵⁹ incorp	g kidney per test rat
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			+	F 55	19 1 ± 2 0	16
3	15	>35		B 64	3 1 ± 1 8	16
			+	C 64	15 8 ± 2 8	16

An arterial constriction was produced by a silver clamp applied to the left renal artery in rabbits

It has previously been reported that in some of the remaining rabbits (group 3) there was an increase in the haemoglobin concentration during the postoperative period (*Hansen* 1964) This increase was pronounced in one case, in which the haemoglobin concentration rose from 12 3 g/100 ml before operation to 17 5 g/100 ml six weeks after operation Increased erythropoiesis was demonstrated in the bone marrow and the erythrocyte volume was distinctly greater in this rabbit than in the other animals We consider that the definite polycythaemia thus demonstrated could be attributed to the application of the clamp to the renal artery

We found it of interest to follow the course of the haemoglobin concentrations in the rabbits in this group In an attempt to return to the conditions prevailing before the increase in haemoglobin, blood was removed from the rabbits when the period of observation had expired until, if possible, a haemoglobin concentration which was slightly below the original value was obtained Subsequently the rabbits were killed to obtain the blood and kidneys for demonstration of erythropoietin *We preferred to produce pooled preparations* of the left and the right kidneys of all animals in the group in order to obtain preparations in which the concentration was as high as possible Consequently, the possibility of establishing whether the demonstrated erythropoietin originated from all or from only a few of the clamped kidneys was eliminated

It was noteworthy that we could not demonstrate erythropoietin in the unfractionated blood plasma, whereas it was present in urine which had been concentrated 8 times In a previous investigation of a patient material we have demonstrated that the erythropoietin content of unfractionated blood plasma is higher than that of urine concentrated 8 times (*Hansen* 1963c) It seems reasonable to explain this finding as the result of the excretion from the clamped kidney of the greater part of the erythropoietin produced in that kidney That part of the erythropoietin which enters the blood might be used in the hyperplastic bone marrow (*Stohlman* 1959) so rapidly that the resulting blood concentration would be too small to be demonstrated by the method used In contrast to this *Nakao et al* (1960) demonstrated "an increased plasma erythropoietic activity in rabbits which underwent the Goldblatt operation by the *in vitro* incubation method"

SUMMARY

The erythropoietin content of urine, plasma, and preparations of pooled kidney extracts has been investigated in rabbits which had undergone Goldblatt's operation An increased erythropoietin content was demonstrated in the urine and in extracts prepared from the clamped kidneys

group 3 The method used did not permit the measurement of the amount of erythropoietin which might have been present (see Table 2)

TABLE 3
Erythropoietin Determination

Clamp	Rabbit no	Test no	Biological response % Fe^{59} incorp
+	9464	*H 62*	3 1 ± 0 85
+	9468	*G 62*	3 2 ± 1 13
—	9465	B 63	3 3 ± 1 96
+	734	*C 63*	3 7 ± 1 20

Unfractionated heparin plasma from rabbits *with renal* arterial constriction was used

DISCUSSION

As the demonstration of an increased erythropoietin content of kidneys with reduced arterial blood supply could throw light on the question of the site of production of erythropoietin, constriction of the left renal artery was carried out in a small group of rabbits (group 1) The right kidney was left untouched so that it could serve as the source of material for the production of preparations which could be used as controls

Only an insignificant blood loss occurred during the operation Consequently, the erythropoietin demonstrated in the urine from the rabbits in group 1 after operation could not have been due to a haemorrhagic anaemia We therefore consider it probable that application of an arterial clamp with a calibre of 0 5 to 0 6 mm conditioned the stimulation of erythropoietin production in, or increased the erythropoietin secretion from, the clamped kidney

The inability to demonstrate erythropoietin in the clamped kidneys could have been due to the fact that the amount of erythropoietin which might have been present in the available kidneys was too small to be demonstrable by the method used, after our mode of preparation Another factor which might have influenced the results was the long time interval between operation and removal of the kidneys It is possible that compensatory mechanisms which would have tended towards a normalization of the compromised oxygen supply to the clamped kidneys could have come into play

The operation was therefore repeated using a larger number of rabbits Some of these lost a great deal of weight during the postoperative period, and were separated as one group (group 2) The interval between operation and the preparation of these kidneys for the demonstration of erythropoietin was shorter than in group 1 More erythropoietin was demonstrated in the clamped than in the contralateral kidneys from this group of rabbits which, however, also contained more individuals than group 1

It has previously been reported that in some of the remaining rabbits (group 3) there was an *increase in the haemoglobin* concentration during the postoperative period (*Hansen* 1964). This increase was pronounced in one case, in which the haemoglobin concentration rose from 12.3 g/100 ml before operation to 17.5 g/100 ml six weeks after operation. Increased erythropoiesis was demonstrated in the bone marrow and the erythrocyte volume was distinctly greater in this rabbit than in the other animals. We consider that the definite polycythaemia thus demonstrated could be attributed to the application of the clamp to the renal artery.

We found it of interest to follow the course of the haemoglobin concentrations in the rabbits in this group. In an attempt to return to the conditions prevailing before the increase in haemoglobin, blood was removed from the rabbits when the period of observation had expired until, if possible, a haemoglobin concentration which was slightly below the original value was obtained. Subsequently the rabbits were killed to obtain the blood and kidneys for demonstration of erythropoietin. We preferred to produce pooled preparations of the left and the right kidneys of all animals in the group in order to obtain preparations in which the concentration was as high as possible. Consequently, the possibility of establishing whether the demonstrated erythropoietin originated from all or from only a few of the clamped kidneys was eliminated.

It was noteworthy that we could not demonstrate erythropoietin in the unfractionated blood plasma, whereas it was present in urine which had been concentrated 8 times. In a previous investigation of a patient material we have demonstrated that the erythropoietin content of unfractionated blood plasma is higher than that of urine concentrated 8 times (*Hansen* 1963c). It seems reasonable to explain this finding as the result of the excretion from the clamped kidney of the greater part of the erythropoietin produced in that kidney. That part of the erythropoietin which enters the blood might be used in the hyperplastic bone marrow (*Stohlman* 1959) so rapidly that the resulting blood concentration would be too small to be demonstrated by the method used. In contrast to this *Nakao et al.* (1960) demonstrated "an increased plasma erythropoietic activity in rabbits which underwent the Goldblatt operation by the *in vitro* incubation method."

SUMMARY

The erythropoietin content of urine, plasma, and preparations of pooled kidney extracts has been investigated in rabbits which had undergone Goldblatt's operation. An increased erythropoietin content was demonstrated in the urine and in extracts prepared from the clamped kidneys.

group 3. The method used did not permit the measurement of the amount of erythropoietin which might have been present (see Table 2)

TABLE 3

Erythropoietin Determination

Clamp	Rabbit no	Test no	Biological response % Fe^{59} incorp
+	9464	H 62	3 1 ± 0 85
+	9468	G 62	3 2 ± 1 13
—	9465	B 63	3 3 ± 1 96
+	734	C 63	3 7 ± 1 20

Unfractionated heparin plasma from rabbits with renal arterial constriction was used

DISCUSSION

As the demonstration of an increased erythropoietin content of kidneys with reduced arterial blood supply could throw light on the question of the site of production of erythropoietin, constriction of the left renal artery was carried out in a small group of rabbits (group 1) The right kidney was left untouched so that it could serve as the source of material for the production of preparations which could be used as controls

Only an insignificant blood loss occurred during the operation Consequently, *the erythropoietin demonstrated in the urine from* the rabbits in group 1 after operation could not have been due to a haemorrhagic anaemia We therefore consider it probable that application of an arterial clamp with a calibre of 0 5 to 0 6 mm conditioned the stimulation of erythropoietin production in, or increased the erythropoietin secretion from, the clamped kidney

The inability to demonstrate erythropoietin in the clamped kidneys could have been due to the fact that the amount of erythropoietin which might have been present in the available kidneys was too small to be demonstrable by the method used, after our mode of preparation Another factor which might have influenced the results was the long time interval between operation and removal of the kidneys It is possible that compensatory mechanisms which would have tended towards a normalization of the compromised oxygen supply to the clamped kidneys could have come into play

The operation was therefore repeated using a larger number of rabbits Some of these lost a great deal of weight during the postoperative period, and were separated as one group (group 2) The interval between operation and the preparation of these kidneys *for the demonstration* of erythropoietin was shorter than in group 1 More erythropoietin was demonstrated in the clamped than in the contralateral kidneys from this group of rabbits which, however, also contained more individuals than group 1

It has previously been reported that in some of the remaining rabbits (*group 3*) there was an increase in the haemoglobin concentration during the postoperative period (*Hansen* 1964). This increase was pronounced in one case, in which the haemoglobin concentration rose from 12.3 g/100 ml before operation to 17.5 g/100 ml six weeks after operation. Increased erythropoiesis was demonstrated in the bone marrow and the erythrocyte volume was distinctly greater in this rabbit than in the other animals. We consider that the definite polycythaemia thus demonstrated could be attributed to the application of the clamp to the renal artery.

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From the Department of Pathology, Section II University of Helsinki, Finland

LIVER REGENERATION IN WHOLE BODY IRRADIATED RATS[1]

By

ANTERO VOUTILAINEN

Received 6 I 64

It has been possible to demonstrate an increase in the number of pathological mitoses by giving whole body irradiation to rats after artificially inducing liver regeneration and mitotic stimulation by CCl_4 or partial hepatectomy The increase was directly proportional to the amount of irradiation (1) *Leong, Pessoti & Krebs* (7) in 1961 noted a 50 60 per cent decrease in mitotic activity in the regenerating liver of experimental animals given 2 courses of 400 r of whole body irradiation from 10 to 8 weeks before the beginning of regeneration About 80 per cent of the ana telophases were abnormal and enlargement was noted in the parenchymal cells

After giving 375 and 600 r of whole body irradiation *Albert & Bucher* (2) established retardation of DNA synthesis and the mitotic phases in liver regenerating after hepatectomy It was most distinct 27 hours after hepatectomy and returned to the level of the controls in 8 weeks

The questions considered here were whether even smaller radiation doses given in the resting phase of hepatic cells before the initiation of regeneration cause fluctuations in the mitotic indices, and whether there are more or fewer pathological mitoses when the irradiation is given earlier or immediately before regeneration In other words, the purpose was to establish whether, when irradiation affects cells which are in the interphase, factors originate that cause changes in the mitotic figures in the initial phase of regeneration and how long they last

MATERIAL AND METHODS

Three month old white male rats (Sprague Dawley) were used for the experiments

[illegible]

irradiated and 1 control group One group was given irradiation alone and 1 was simply a control group

[1] Supported by the Sigrid Juselius Foundation and Damon Runyon Memorial Fund (DRG 644)

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TABLE 1

Mitotic and Weight Index of the Liver after Hepatectomy when Irradiation is Given Earlier than 7 Days after Hepatectomy Each Group Includes 10 Animals

Mitotic index (number of mitoses per 0 785 sq mm)
Mean ± Standard error

Time after hepatectomy	Control		Irradiation		Irradiation and hepatectomy		Hepatectomy	
	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitosis	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses
2 days	2 0 ± 1 34	0	1 2 ± 0 44	0 4	16 6 ± 3 18	8 6	10 8 ± 1 16	3 0
14 days	1 2 ± 0 9	0	1 2 ± 0 44	0 4	2 7 ± 0 59	1 6	1 8 ± 0 39	0 2

Weight index $(1000 \times \frac{\text{liver weight}}{\text{body weight}})$

Mean + Standard error

Time after hepatectomy	Control	Irradiation	Irradiation and hepatectomy	Hepatectomy
2 days	38 3 ± 2 7	39 5 ± 2 2	26 7 ± 2 8	26 2 ± 2 5
14 days	41 7 ± 4 8	42 4 ± 5 9	42 2 ± 4 7	38 6 ± 6 9

Partial hepatectomy was performed under ether anaesthesia The median and left lobes (70 per cent) were removed by the method of *Higgins & Anderson* (5) The animals were weighed during irradiation and at the time of decapitation 2 days after hepatectomy Both measures were performed at 2 o'clock p m Pieces of tissue were taken from the liver at the time of decapitation, fixed in Bouin's solution and stained with haemalum eosin Mitoses were counted per 100 visual fields with Wild s binocular microscope, magnification 10 × 100 × 1 5, totaling an area of 0 785 sq mm The liver weights were recorded at the time of decapitation and the weight indices were calculated from the formula $1000 \times \frac{\text{liver weight}}{\text{body weight}}$ (6, 14)

The statistical analyses were performed by Student's t-test (3)

In the second series the animals were distributed into similar groups The only difference was that hepatectomy was performed immediately after whole body irradiation and the animals decapitated 2 and 14 days after hepatectomy at 2 o clock p m Mitotic and weight indices were determined as above

RESULTS

Irradiation followed by hepatectomy after 7 days The mitotic index of the irradiated group was at the level of the controls both after 2 and 14 days (Table 1) The mitotic index rose in the usual way in 2 days in the hepatectomy group and was at the normal level after 2 weeks In the irradiation-hepatectomy group it was even higher 2 days after hepatectomy and back to normal after 2 weeks, pathological mitoses in this group totalled 55 per cent and in the hepatectomized group 28 per cent

The weight index rose in the irradiated group ($P < 0\ 001$) 9 (7 + 2) days after irradiation but then fell to the control level in 14 days In both hepatectomized groups the index rose to normal in 2 weeks (Table 1)

Irradiation and hepatectomy on the same day The post-irradiation mitotic index was at the control level within 2 and 14 days, but in the group treated with irradiation and hepatectomy it was even higher 2 days after hepatectomy, than in the corresponding group of the first series, 47 per cent of the mitoses being pathological The mitotic index returned to normal in this and in the other groups after 14 days when approximately half of the very few mitoses seen were pathological (Table 2)

The weight indices were at the normal level in the irradiated group The post-hepatectomy weight indices rose similarly both in the irradiated group and in the group treated with hepatectomy alone

DISCUSSION

The present study shows that whole body irradiation causes changes in the regeneration of the liver which are seen both in mitotic counts and changes in liver size Artificially increased mitotic count of the liver by hepatectomy was increased further 2 days after hepatectomy by a dose of 250 r administered 7 days earlier The mitotic rate increased even more vigorously when the irradiation and hepatectomy



Fig 1

A telophase bridge 2 days after irradiation and 70 per cent hepatectomy (Magnification 5460 ×)

were performed on the same day The second point to be noted is that pathological mitoses occur in both of these groups, reaching about 50 per cent 2 days after hepatectomy Thirdly, irradiation does not change *mitotic activity in the liver without artificial mitotic stimulation* This confirms the earlier experiments with doses of 200 r or more (10, 11) However the weight index is significantly increased when irradiation is given 7 days before hepatectomy Fourthly 14 days after hepatectomy the mitotic index and weight index returned to the level of the controls in all the groups However pathologic mitoses still occured in half of the few mitoses in the irradiated groups

In earlier studies by *Long et al* (7) a decrease in mitotic activity was established as a late effect in c 50 60 per cent of the material An increase was manifest in the present work both when irradiation was given immediately and when given a week before the beginning of regeneration Pathological mitoses weer more numerous than *Albert's* (1) experiments in which regeneration was induced by means CCl_4 and the mitoses were counted only in the ana telophases immediately before and after irradiation Usually after hepatectomy the ionizing radiation decreases the mitotic index definitely (15)

Pathological mitoses displayed *e g* clumping of chromosomes between the separated chromosomes (Fig 1) bridge mitoses There were also chromosome bundles that separated in the prophase (Fig 2) and *an aberrant chromosome could remain* separate between the separated chromosomes (Fig 3) This is usually regarded as damage caused by a small radiation dose According to *Teir* (12 13) the result of such mi

TABLE 2

Mitotic and Weight Index of the Liver 2 and 14 Days after Irradiation and 70 per Cent Hepatectomy

Mitotic index (number of mitoses per 0 785 sq mm)

Mean + Standard error

Time after hepatectomy	Control		Irradiation		Irradiation and hepatectomy		Hepatectomy	
	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses	Normal mitoses	pathol mitoses
2 days	2 0 ± 1 34	0	1 1 ± 0 09	0 5	18 9 ± 1 74	8 7	10 8 ± 1 16	3 0
14 days	1 2 ± 1 3	0	0 9 + 0 38	0 4	1 7 ± 0 80	0 8	1 8 ±0 39	0 2

Weight index $(1000 \times \frac{\text{liver weight}}{\text{body weight}})$

Mean ± Standard error

Time after hepatectomy	Control	Irradiation	Irradiation and hepatectomy	*Hepatectomy*
2 days	38 3 + 2 7	39 5 ± 2 2	26 7 + 2 8	26 2 ± 2 5
14 days	41 7 ± 4 8	42 4 + 5 9	42 2 + 4 7	38 6 ± 6 9



Fig 1

A telophase bridge 2 days after irradiation and 50 per cent hepatectomy (Magnification 460 ×)

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TABLE 2

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Mitotic index (number of mitoses per 0 785 sq mm)

Mean ± Standard error

Time after hepatectomy	Control		Irradiation		Irradiation and hepatectomy		Hepatectomy	
	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses
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Mitotic and Weight Index of the Liver 2 and 14 Days after Irradiation and 70 per Cent Hepatectomy.

Mitotic index (number of mitoses per 0.785 sq mm)
Mean ± Standard error

Time after hepatectomy	Control		Irradiation		Irradiation and hepatectomy		Hepatectomy	
	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses	Normal mitoses	Pathol mitoses
2 days	2.0 ± 1.34	0	1.1 ± 0.09	0.5	18.9 ± 1.74	8.7	10.8 ± 1.16	3.0
14 days	1.2 + 1.3	0	0.9 ± 0.38	0.4	1.7 + 0.80	0.8	1.8 ± 0.39	0.2

Weight index $\left(1000 \times \frac{\text{liver weight}}{\text{body weight}}\right)$

Mean ± Standard error

Time after hepatectomy	Control	Irradiation	Irradiation and hepatectomy	Hepatectomy
2 days	38.3 ± 2.7	39.5 ± 2.2	26.7 ± 2.8	26.2 ± 2.5
14 days	41.7 ± 4.8	42.4 ± 5.9	42.2 ± 4.7	38.6 ± 6.9



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Fig 4
The liver tissue 9 days after irradiation and 2 days after 70 per cent hepatectomy (Magnification 1820 ×)

totic disturbances is not 2 daughter cells but only one macronuclear cell which cells in fact were encountered in the irradiated liver tissue

Irradiation effects have been found even after the smallest doses, not only as changes in the size of the nucleus but also as swelling of the cells (4, 10) and as the dose increases, as necroses in the liver parenchyma (4) These phenomena were manifest in the present work as increase in the intercellular substance and such a degree of oedema in the cytoplasm that the cell boundaries were not clearly visible (Fig 4) These changes are demonstrable more clearly when at least 7 days have elapsed after irradiation The enlargement of the liver is so definite that the increase in the weight index is highly significant in the irradiated group However after 2 weeks the weight index is again at the control level obviously because of the small size of the dose The liver is not resistant to ionizing radiation as proven by changes in the liver cells provoked by 250 r as well as by *Scherer's* (10) studies with whole body irradiation 400 r Contrary views have also been advanced (9) even after doses 600 2500 r to the liver According to *Gershbein*, as much as 3200 20000 r after partial hepatectomy did not change the regeneration of liver significantly (4) However, numerous necroses were established histologically in the liver parenchyma

In the present work, too the irradiated and hepatectomized test animals did not fall behind the controls in liver regeneration judging by the liver weight indices *Albert et al* (2) and other authors have

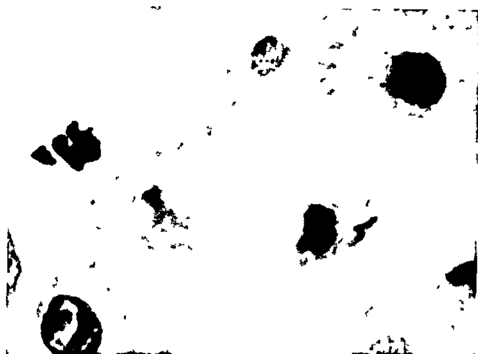


Fig 2
The separated chromosome bundles of two mitoses 9 days after irradiation and 2 days after 70 per cent hepatectomy (Magnification 2380 ×)

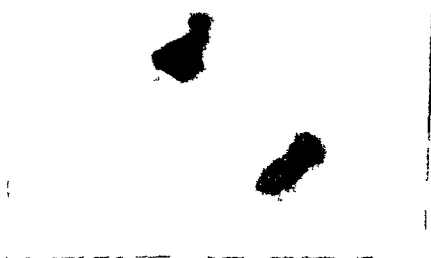


Fig 3
An anaphase 2 days after irradiation One aberrant chromosome is situated between the separated chromosomes (Magnification 4550 ×)

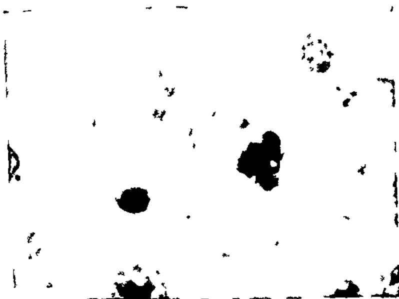


Fig 4
The liver tissue 9 days after irradiation and 2 days after 0 per cent hepatectomy (Magnification 180 ×)

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An anaphase 2 days after irradiation One aberrant chromosome is situated between the separated chromosomes (Magnification 4550 ×)



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The liver tissue 9 days after irradiation and 2 days after 70 per cent hepatectomy (Magnification 1820 ×)

totic disturbances is not 2 daughter cells but only one macronuclear cell which cells in fact were encountered in the irradiated liver tissue

Irradiation effects have been found even after the smallest doses, not only as changes in the size of the nucleus but also as swelling of the cells (4, 10) and, as the dose increases, as necroses in the liver parenchyma (4) These phenomena were manifest in the present work as increase in the intercellular substance and such a degree of oedema in the cytoplasm that the cell boundaries were not clearly visible (Fig 4) These changes are demonstrable more clearly when at least 7 days have elapsed after irradiation The enlargement of the liver is so definite that the increase in the weight index is highly significant in the irradiated group However, after 2 weeks the weight index is again at the control level, obviously because of the small size of the dose The liver is not resistant to ionizing radiation, as proven by changes in the liver cells provoked by 250 r as well as by *Scherer's* (10) studies with whole body irradiation 400 r Contrary views have also been advanced (9) even after doses 600–2500 r to the liver According to *Gershbein*, as much as 3200 20000 r after partial hepatectomy did not change the regeneration of liver significantly (4) However, numerous necroses were established histologically in the liver parenchyma

In the present work, too, the irradiated and hepatectomized test animals did not fall behind the controls in liver regeneration judging by the liver weight indices *Albert et al* (2) and other authors have



Fig 2
The separated chromosome bundles of two mitoses 9 days *after irradiation* and 2 days after 70 per cent hepatectomy (Magnification 2380 ×)

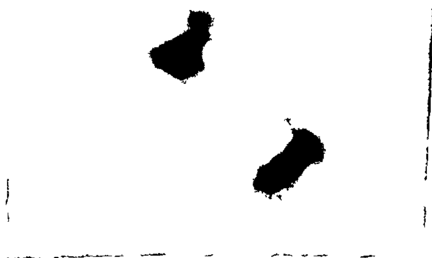


Fig 3
An anaphase 2 days after irradiation One aberrant chromosome is situated between the separated chromosomes (Magnification 4550 ×)

9 Lohle F A & Buling C H Acta Rad 1 13 117 1932
10 Scherer F Strahlentherapie 100 86 1956
11 Spla S Acta path et microbiol scandinav., Suppl 141 1960
12 Teir H Acta path et micr biol scand nav Suppl 51 1944
13 Teir H Soc Scient Fenn., Comm Biol XIII 6 1 1951
14 Teir H & Lahtiharju A Exp Cell Res 24 424 1961
15 Williams R B Jr DeLong R P & Jaffe J J Am J Path I 8 546 1952

established a retardation within 27 hours of DNA synthesis and the mitotic phases The gap was reduced to normal in 8 weeks This is probably due to the increase in the number of pathological abnormal mitoses, for instance in the present work up to 55 per cent in 2 days This means that the cell-division time has been lengthened and the life span of the cell thus prolonged Using H^3-thymidine labelling, *McDonald* noted that the average life span of cells in the regeneration process is 2 or 5 times longer than that of the cells of non-irradiated rats (8) Thus, in addition to an increase in the number of abnormal mitoses irradiation of cells before the institution of the regeneration process causes also a super-normal incidence of mitoses in the regeneration phase on account of the lengthening of the cell division time

SUMMARY

Three-month-old male rats were distributed into groups of 10 animals Whole body irradiation, 250 r, was given to 2 groups and 70 per cent hepatectomy was performed 7 days later on one group and one control group The rats were decapitated 2 and 14 days later and the liver mitoses were counted in an area of 0 785 sq mm The weight indices of liver at the time of decapitation were recorded In a second experiment irradiation was given immediately before hepatectomy but otherwise the procedure was as described above

In the non-hepatectomized rats given irradiation the mitoses remained throughout at the level of the controls, but in both groups treated with irradiation plus hepatectomy mitoses were nine numerous 2 days after hepatectomy than in the non-irradiated hepatectomized group Within 2 days pathological mitoses accounted for nearly one half of all of the mitoses in the irradiated hepatectomized group The weight index rose highly significantly in the irradiated non hepatectomized group when irradiation had been administered 7 + 2 days earlier Macronuclear cells and swelling of cells were observed in the irradiated liver in consequence of the mitotic disturbances

It is probable that irradiation to cells before the start of regeneration causes not only an increase in abnormal mitoses but also an abnormally incidence of mitoses in the initial phase of regeneration since the cell division time has been prolonged

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WHIPPLE'S DISEASE ASSOCIATED WITH AMYLOIDOSIS

By

STEN SANDER

Received 10 i 64

Even today, Whipple's original *description* (1907) covers most of the symptoms, signs and pathological lesions characteristic of the disease bearing his name The main clinical features are diarrhoea with malabsorbtion, polyarthralgia (often migratory), and pigmentation of the skin.

Whipple proposed the name "intestinal lipodystrophy" and attention was for a long time focussed on the accumulation of lipid and large macrophages with a "frothy" cytoplasm present in the intestinal submucosa Additional information was obtained by *Black-Schaffer* (1949) who demanstrated non-lipid substances within the macrophages of typical lesions using the periodic acid Schiff (P A S) reaction *Sieracki* (1958) first called attention to the frequent distribution of the P A S positive material in sickle-formed particles within the macrophages (Sickle-particle cells, S P C)

The etiology of Whipple's disease and the origin of the sickle-formed particles is still obscure The many points of similarity between this disease and storage diseases have led several authors to suggest a similar pathogenesis On the other hand Whipple's original report as well as publications during the last few years (2, 13) emphasize the possible aetiological rôle of micro organisms Bacteria-like organisms have been observed within macrophages as well as in tightly packed extracellular clusters in the submucosa of the small intestine and in the mesenteric lymph nodes

A coincidence of Whipple's disease and amyloidosis may be of peculiar interest, since abnormal deposits of gluco proteins are observed within the reticulo-endothelial cells in both conditions

The present report deals with the coincidence of Whipple's disease and amyloidosis an association which has not been reported previously

CASE REPORT

A 47 years old farmer was first seen in june 1962 complaining of abdominal cramps and diarrhoea He had experienced a progressive weight loss for the last year, and the stools had been numerous watery and malodorous Clinical examination revealed pigmentations of the skin general enlargement of lymph nodes and hypochrome anaemia A diffuse tenderness was recorded over the abdomen but there

X-ray examinations of the small bowel revealed "defici ... fat

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Autopsy (RH O 18263)

The intestines appeared essentially normal, but the mucosa of the small intestine looked hypertrophic The lymph nodes were generally swollen, the enlargement being most pronounced in the mesentery and in the retroperitoneal tissue The lymph nodes were firm and well defined the cut section of some mesenteric nodes were porous looking No signs of necrosis nor calcification were present No obstructive lesions were detected along the thoracic duct or its tributaries and ascites was not present *Furthermore, left sided pleuritis, fibrinous* pericarditis and bronchopneumonia were recorded

Histological Study

Tissue for microscopical examination was fixed in formalin and embedded in paraffin The following staining procedures were performed haematoxylin-eosin Congo red periodic acid Schiff (P A S) with and without diastase digestion Toluidine blue, Methyl violet Alcian blue and Ninhydrin Schiff Fat stains were performed on frozen sections Bacteria were looked for in Gram and Loeffler stained sections

Sections from the small intestine revealed swollen and partly club-shaped intestinal villi The surface cells appeared normal The villi were distended and distorted by closely packed cells containing typical sickle-formed particles Fig 1 In addition P A S positive nonlipid substance was demonstrated as amorphous and granular strands in the submucosa and the mesenteric lymph nodes A slight infiltration of lymphocytes and plasma cells and of lipid containing "foam cells" was likewise observed

Within the mesenteric lymph nodes the architecture was deranged and a definite accumulation of fat was observed In some of the swollen lymph nodes small nests of epitheloid cells with a few giant cells were noticed In addition to the numerous S P C cells large *amorphous* deposits were observed in the mesenteric and retroperitoneal lymph nodes Fig 2 Amyloid was suspected and confirmed by Congo red and methyl violet stainings Metachromacy was distinctly observed after methyl violet staining and *polaroscopy disclosed dichroism* in Congo red stained sections Amyloid deposits were also present in the thyroid

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were no palpable masses X ray examinations of the small bowel revealed "deficiency pattern" and malabsorbtion was demonstrated by the xylose test and by fat determinations in the faeces The patient was discharged with the diagnosis of sprue and he was controlled at regular intervals When migratory polyarthritis pleuritis and dyspnoea supervened Whipple's disease was considered He was given supportive treatment blood transfusions and prednison The illness ran a febrile course ... state ... ... sero ... ... nages ... ... dis

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Fig 2

Amylo d d pos ts in mes nter c l mph n d and large macro hages c nta ning I A S po t e sul tan e I A S × 140



Fig 3

I a a ke organ ms n ma r phag s f om mes n r c lymph node I A S × 1200



Fig 1
Club shaped intestinal villus with tightly packed macrophages containing P A S positive substance P A S × 120

gland, adrenals, kidneys, liver, spleen, and most of the lymph nodes examined Stains for bacteria disclosed smaal rod-shaped particles compatible with bacteria within the macrophages as well as in the submucosa of the small intestine Fig 3. The sickle-formed particles were Gram positive and showed a blue-green colour in Loeffler stained sections The Ziehl-Nielsen staining for mycobacteria was negative

DISCUSSION

The present case demonstrates the clinical as well as the pathological characteristics of *Whipple's disease The histochemical properties* of the sickle-formed particles (Table 1) are essentially the same as those reported by others (6, 7, 11) With attention focussed on the P A S positive particles essential for the diagnosis, two possible explanations should be considered as to their origin (5)



Fig 2

Amyloid deposits in mesenteric lymph node and large macrophages containing P A S positive substance P A S × 140



Fig 3

Bacteria like organisms in macrophages from mesenteric lymph node P A S × 1200



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Fig 2

Amyloid deposits in mesenteric lymph node and large macrophages containing P A S positive substance P A S × 140



Fig 3

Bacteria like organisms in macrophages from mesenteric lymph node P A S × 1200

1) The first possibility is that the P A S. positive substance including the sickle-formed particles might be the result of cytoplasmatic elaboration of abnormal glucoproteins Based on extensive studies on experimental amyloidosis *Teilum* (1956) has expressed the opinion that reticulo-endothelial cells containing P A S positive substance may be directly concerned in the local formation of amyloid In fact, Teilum has described polysaccharide containing globulins and granules formed in the cytoplasm during the stage of amyloid precipitation According to this view the present case might be interpreted as a demonstration of disturbed protein synthesis of reticulo-endothelial cells with resulting production of sickle-formed particles and amyloid These gluco-proteins might be produced in response to antigenic stimulation by the micro-organisms found locally. Amloid and sickle-formed particles have, however, distinctly different staining properties and althoug these substances are found within the same lymph nodes they are not observed within the same macrophages in our case

TABLE 1

	Hyaline substance (amyloid)	S P C cells
P A S	+	+++
Diastase/P A S	+	+++
Methyl violet	metachromacy	violet
Congo red	+++	—
Sudan III	—	—
Ninhydrin Schiff	+	+ (weak)
Toluidine blue	blue	b metachromacy
Alcian blue	—	—

2) *Phagocytosed microorganisms The result of the bacterial staining* performed in our case support the view that these structures may be of bacterial origin The granular masses found together with the rod-shaped structures may represent partly digested bacteria The strong P A S *positive reaction may be due to muco polysaccharides* coating the bacterial surface In our case microorganisms morphologically similar to those found at autopsy have been demonstrated by electron microscopy in a biopsy taken one month before death (*Kjaerheim*, to be published)

The bacteria-like structures observed make an infectious aetiology most probable Reports of improvement and control of diarrhoea (2, 4) after treatment of Whipple's disease with antibiotics support this view The local inflammatory changes observed in our case might so far be in accordance with this concept The amyloid deposits in this case were situated in the thyroid gland, adrenals, kidneys, liver, spleen and lymph nodes, a distribution compatible with secondary amyloidosis According to current opinion corticoid treatment, which was given to our

patient during several months, may accelerate the deposition of amyloid

The possibility exists, however, that the two different aspects discussed may both be pertinent in Whipple's disease Elaboration of abnormal gluco-proteins within the cytoplasm of reticulo-endothelial cells might be induced by antigenic stimulation from the phagocytosed microorganisms observed in the small bowel Identification of the microorganisms in question, and their ultimate fate in the diseased tissue requires, however, further investigations

SUMMARY

A case of Whipple's disease associated with amyloidosis is reported Large macrophages containing P A S positive substance and bacteria-like structures were disclosed in the submucosa of the small intestine and in the mesenteric lymph nodes Amyloid was found in the same mesenteric lymph nodes and in several other organs The pathogenesis is discussed with particular attention paid to the bacteria-like structures and the abnormal gluco-proteins observed The hypothesis is considered that elaboration of abnormal globulins within the reticulo-endothelial cells might be induced by antigenic stimulation from the microorganisms observed in the small bowel A plea is made for routine amyloid examinations and for identification of microorganisms in Whipple s disease

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QUANTITATIVE ESTIMATION OF THE MAST CELLS IN THE DERMAL CONNECTIVE TISSUE OF HAEMOPHILIC PATIENTS

By

KNUD ERIK SJØLIN

Received 1 la i 64

In two earlier published works the occurrence of metachromasia in the dermal connective tissue from patients with haemophilia was described (*Sjølin* 1951 *Sjølin* 1962)

In the present work an account is given of the results of counting the mast cells in the dermal connective tissue from 7 patients with haemophilia and a number of control patients without signs of haemorrhagic disorders

METHODS

The b psies from the patients were taken with a cutting punch on the back f the upper arm ab ut 2 inches ab ve the tip of olecran n The fresh bi psies had a diameter fr m 2 to 4 mm and c mprised the epidermis derma and subcutis The defect in the skin was sutured with catgut on an atraumatic needle The suture was removed 10 days after the operati n

The preparation f sl des was standardize l as f ll ws

1 Fixation was performed in 4 per cent basic soluti n of lead acetate f r 24 h urs (*Hellstrom & Holmgren* 1950)
2 Rins ng in distilled water f r 5 minutes
The bi psy was then placed in
3 Seventy per cent ethyl alc h l for 1 h ur
4 N nety six per cent ethyl alc h l for 1 h ur
5 Ninety n ne per cent ethyl alcohol f r two hours
6 Xylene for 1 hour
7 Melted paraffin wax for 12 h urs
8 Embedded in paraff n wax

The embedded biopsies were then cut in 96 μ th ck secti ns and placed in a ... eparaffining an l the stain ... l alc hol 2 times 2 minutes ... c hol 1 minute 2 per cent ... on of t luidine blue (pH 5) f r 1½ minutes Different ation in a solution of 10 per cent f rmaldehyde 96 per

In estimating the number of mast cells the follow ng procedure was used The specimen was placed so that the visual field reached the deeper border of the

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TABLE 4

The Number of Mast Cells on the Back of the Left Arm and on the Left Thigh, 2 Inches above the Left Knee

Age	Left arm	Left thigh
4 months	2153	1949

TABLE 5

Number of Mast Cells in Control Material

Age	Total	Superficial field	Deep field
1 1 day	2363	1250	1113
2 40 days	1840	961	879
3 4 months	2153	1290	863
4 4 months	2297	1401	896
5 8 months	2216	1152	1064
6 11 months	2433	1341	1092
7 13 months	1902	1020	882
8 1 year 2 months	2206	1404	802
9 1 year 2 months	1497	831	666
10 1 year 11 months	1914	1230	684
11 3 years 1 month	1620	743	877
12 5 years	1206	595	611
13 7 years 10 months	1266	707	559
14 13 years	274	161	113
15 15 years 8 months	976	516	460
16 17 years	968	569	399
17 18 years 10 months	261	152	109
18 19 years 11 months	939	512	427
19 22 years	1038	641	397
20 23 years	1062	5a7	505
21 23 years 8 months	1114	441	673
22 23 years 10 months	1330	686	644
23 24 years 3 months	1212	762	450
24 26 years	660	391	269
25 26 years	1157	711	446
26 28 years	779	483	296
27 30 years	652	386	266
28 30 years 4 months	1321	539	782
29 30 years 5 months	1150	628	522
30 36 years 7 months	741	346	395
31 38 years 11 months	650	365	285
32 40 years 6 months	1017	679	338
33 41 years 5 months	978	467	511
34 43 years 5 months	1050	644	406
35 45 years 9 months	689	376	313
36 48 years 2 months	938	551	387
37 50 years	674	319	355
38 50 years	975	433	542
39 50 years 9 months	1944	1229	715
40 51 years 7 months	925	498	427
41 52 years 1 month	756	371	385
42 56 years	558	269	289
43 56 years 8 months	1196	685	511
44 60 years	434	223	211
45 61 years 5 months	601	303	298
46 62 years 8 months	1087	557	530
47 64 years 7 months	933	560	373
48 71 years 5 months	980	578	402

epidermis, after which the microscope tube was raised and lowered in order to enable the observer to count all the mast cells situated in the entire field within the thickness of the section (superficial field). The position of the specimen was then altered so that the field deeper in the corium and subcutis touching the lower limit of the preceding field could be examined (deep field). The number of mast cells with visible nuclei was estimated in 5 sections using every second of ten consecutive, from each tissue sample. The mast cells were counted in 3 superficial and 3 deep fields of each section, that is 30 fields in all from every patient.

Leitz microscope with an ocular of 12 × and objective 40 × (8 mm) was used. During elaboration of the method it was demonstrated, that application of formol fixation in stead of lead acetate fixation of the tissue samples, reduced the number of mast cells to about one third at least (Table 1).

TABLE 1

	Fixation	
	Lead acetate	Formol
Number of mast cells	1944 1196	341 335

MATERIAL

As mentioned before the material consisted of skin biopsies from 7 haemophilic patients. The number of mast cells in this material was compared with the number of mast cells in skin necropsies taken in the same region.

The skin samples from the autopsy material were taken within 72 hours after the death of the patients. According to *Hellstrom & Holmgren* (1950) storing of skin tissue at +4° C during 72 hours does not alter the number of mast cells significantly.

In order to obtain an impression of the variation of the number of mast cells in the same person skin samples were taken from different sites. Table 2 shows the variations in the number of mast cells ca. 1, 2 and 3 inches above the tip of olecranon respectively.

TABLE 2

Variations in the Number of Mast Cells at Different Sites of the Left Arm

Age	Inches above the tip of olecranon		
	1	2	3
½ year	2297		1927
1 year 2 months	1389	1497	
18 years 10 months	275	261	299
19 years 11 months	675	939	795
23 years 8 months	1114		1286
23 years 10 months	1330		1201
30 years 5 months	1150		1116
36 years 7 months	579	741	792
43 years 5 months	1302	1050	1440
56 years		558	601
60 years	*391*	434	

TABLE 3

The Number of Mast Cells on the Back of the Arm 2 Inches above the Tip of Olecranon

Age	Left arm	Right arm
3 years 1 month	1620	1626
22 years	1038	1122
52 years 1 month	756	606

The number of mast cells in the haemophilic patients is seen in Table 6 as well as the clotting defects and the occurrence of metachromasia. The number of mast cells was plotted in the double logarithmic system too. It is seen that the number of mast cells in the controls and in the haemophilic patients were of the same magnitude.

The number of mast cells in the deeper part of corium alone was compared in the two groups. Neither in this part of the skin could any difference be demonstrated.

DISCUSSION

Several investigators have demonstrated a decrease in the number of mast cells with advancing age (*Brack* 192?, *Fischer* 1937, *Hellstrom & Holmgren* 1950, *Sundberg* 195?, *Iversen* 1960). The finding in the present experiments is in accordance with those. The number of mast cells in the dermal connective tissue and subcutis from haemophilic patients did not differ from the mast cell numbers in the controls.

The number of mast cells in biopsies from haemophilic patients with metachromasia did not differ from the mast cell number in other biopsies.

In two of the controls 13 and 19 years old respectively the number of mast cells was remarkably low compared with the rest of the controls. These two patients suffered from a congenital heart disease and melanocarcinoma respectively.

SUMMARY

The number of mast cells counted in 96 μ thick sections in the dermal connective tissue of haemophilic patients was compared with that of controls. Whether the connective tissue in the haemophilic patients had metachromasia or not the number of mast cells did not differ from the controls.

The number of mast cells decreased with age both in the haemophilic patients and the controls. In two controls patients with congenital heart disease and melanocarcinoma respectively the number of mast cells was remarkably low.

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TABLE 6
Patients with Haemophilia

Family	Patient no	Age in years	Deficiency in clotting system	Number of mast cells	Superficial field	Deep field	Metachromasia
1	9	1	AHF (VIII) + Christmas IX	1695	811	884	strong
4	10	5	Christmas (IX)	1071	505	566	strong
72,	1	6	AHF (VIII)	1502	752	750	weak
66	12	12	AHF (VIII)	936	502	434	none
68	1	13	AHF (VIII)	1194	543	651	distinct
2	15	17	AHF (VIII)	1120	544	573	distinct
51	3	32	Christmas (IX)	1003	520	483	weak

It was found that the variation in the number of mast cells varied considerably in the different persons

In three persons the number of mast cells in the skin on the back side of the arm 2 inches above the tip of the olecranon was compared (Table 3) The number of mast cells was of the same magnitude, but again there was some variation in the same person

In one person the number of mast cells on the back of the left arm was compared with the number of mast cells 2 inches above the left knee (Table 4) Again there was some variation in the number of mast cells but the number was of the same magnitude

RESULTS

Table 5 demonstrates the results of counting mast cells in 48 persons without known disturbances in the clotting system

In Fig 1 the number of mast cells is plotted against the age in a double logarithmic system In the age groups 0 2½ years, 2½ 5 years etc the average number of mast cells was calculated

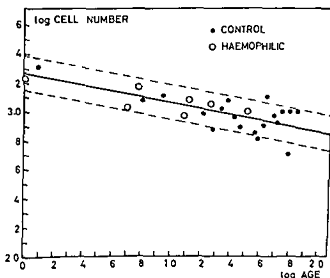


Fig 1
The number of mast cells in controls in age groups and in haemophilic patients plotted in a double logarithmic system
The dotted lines indicate the standard deviation

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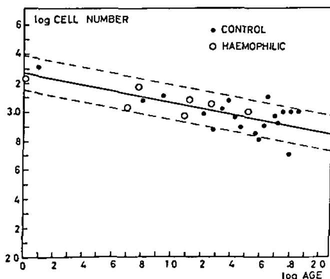


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SIGNIFICANCE OF CHROMOSOMAL INVESTIGATION OF SOMATIC CELLS TO DETERMINE THE GENETIC ORIGIN OF GONADOBLASTOMA

(*Gonocytoma III*)

By

JOHN PHILIP and JERZY TETER

Received 20 II 64

Patients with congenital somatosexual ambiguities have a tendency to develop gonadal neoplasms (7, 13). The risk of tumours developing in abnormal gonads is relatively high. One of us (J T) found neoplastic changes in four out of 13 consecutively operated cases of primary amenorrhoea with somatosexual disturbances (14). *Morris* (8) found that in 82 cases of testicular feminization seven or almost 10 per cent developed tumours. *Dixon & Moore* (4) found that the risk of tumours in an undescended testis in otherwise normal males is ten times as high as in a descended. Other authors found the frequency of tumours in ectopic testes as high as 48 times of the risk in a scrotal testis. The risk seems higher in an abdominal than in an inguinal testis.

In cases of testicular dysgenesis in individuals with characteristics of male pseudo-hermaphroditism germ cell tumours are most frequently found. Three main factors may be of importance: a) a permanent pituitary hyperactivity (confirmed by the high FSH level in these patients), b) dystrophic background consisting of a rudimentary and malformed gonad, c) persistent germ cells (gonocytes) from the embryonic stage.

Teter (15) divides the gonocytoma tumours in 4 types: gonocytoma I, II, III and IV. This paper only discusses the gonocytoma III, which is identical with *Scully's* gonadoblastoma.

The gonocytoma III tumours seems to occur frequently in patients with external genitals of female type but with a male chromosome complement (with a Y chromosome) (testicular dysgenesis). *Gardner*

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The expert technical assistance of *Bente Møller* is greatly appreciated



Fig 1 a

(6) 1958 assumed that the male enviroment favours the growth of the neoplastic cell in experimental ovarian tumours. This experimental evidence has been corroborated by clinical findings. Evidence obtained from the present studies support the above mentioned view.

It must here be mentioned that the four tumours found by *Teter & Tarlowski* (14) *in primary amenorrhoea were unexpected by the time* of operation. Because of the tumour-risk as well as for the importance of establishing a diagnosis, there can be no doubt that all chromatin-negative patients, especially when the chromosome-complement is XY with primary amenorrhoea should have an exploratory laparotomy.

The classification of gonadal tumours is difficult and will not be discussed from the histological point of view in this paper. It must be stressed, however, that *Teilum* (10) (11) *has produced evidence* that certain gonadal tumours in the male and the female are identical and homologous.

It is therefore difficult with certainty to distinquish between male and female elements in gonadal tumours, especially of embryological origin. The difficulty is even greater in cases where the tumours arise in patients with intersexuality.

One rare tumour type in which the problem of the genetic origin of the elements is very obvious in the so-called gonadoblastoma of *Scully*, or in *Teter's* classification of germ cell tumours — gonocytoma *III*.

The gonadoblastoma was first described by *Scully* (9) who reviewed a series of gonadal tumours *earlier classified as dysgerminomas or ar-*

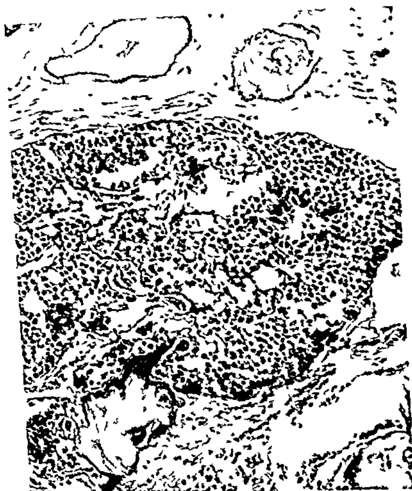


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Gonadoblastomas usually occur in patients with 1) a female phenotype 2) signs of masculinization and 3) a negative sex chromatin pattern

The tumour is histologically characterized by the presence of a heterogenous mixture of cellular elements germ cells as well as Sertoli granulosa like cells and interstitial Leydig like cells The neoplastic nests are composed of germ cells scattered among Sertoli granulosa cells The latter are arranged in three typical patterns described by *Scully* 1) in "coronal fashion around germinal cells 2) in peripheral



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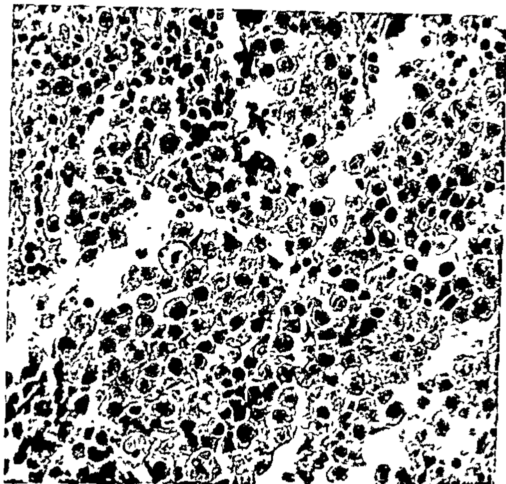


Fig 1 c

Fig 1 a b and c
Microphotography of histological sections of a gonadoblastoma
See text for explanation

single file along the base of the pseudotubules, d) in folliculoid pattern (Fig 1 a) Foci of Leydig cells are clustered between the cellular nests described above In many areas microscopic examination shows in the hyalinized stroma small groups of Leydig cells and calcified concretions in form of irregular masses or "psammona-like" bodies (Fig 1 b) In the other parts the neoplastic nests form "pure dysgerminoma" (Fig 1 c)

Pathologists agree that a definite histological assessment of the type of gonad, in which the tumour arise, is not possible, and *Scully* has indeed stressed in his first paper that "not only are we ignorant of exactly what gonadal elements the tumour are differentiating towards, but we also cannot say with certainty from what type of gonad the tumour arose".

As earlier mentioned patients with this tumour are believed always to be chromatin-negative and all *Teter's* cases have been shown to be so

		< 43	43	44	45	46	47	Total
W 1	Blood	5	1	5	10	52	2	75
	Skin							
W 2	Blood	2		7	32	3		44
	Skin	1		3	34	11	1	50
W 3	Blood							
	Skin			2	4	24		30
W 4	Blood		1	2	3	24		30
	Skin	1			2	26	1	30
W 5	Blood							
	Skin	1			2	28		31

Fig 2

Chromosomal counts in 5 cases of *male pseudohermafroditism* with gonadoblastoma

Scully's cases were published in 1953, when examination of presence of Barr bodies in humans were rarely done and the case of *Fine et al*, (5) was chromatin-negative *Borghi* and co-workers described in 1962 (2) a case of gonadoblastoma (gonocytoma III) in a 11-years old "female" with precocious isosexual puberty followed by virilization Her sex chromatin pattern was also negative. But chromatin-negativity only means that the patient has not two normal X-chromosomes and does not give the final information about the genetic sex of the patient

Examination of the chromosomal sex of the five patients of *Teter* has been carried out and Fig 2 shows some of the results

In all but one case the examined somatic tissues had 46 chromosomes In the fifth (W2) the blood had 45 (and 46?) chromosomes, the skin was a mosaic consisting of cells with 45 and 46 chromosomes In all cells from the 4 cases with 46 chromosomes the chromosome-complement was found to be compatible with that of a normal male, that is 22 pairs of normal autosomes and 2 sex-chromosomes, *i e* XY In the case of mosaicism, the cells with 46 chromosomes had the male complement, while the cells with 45 chromosomes had 15 chromosomes in the 6-12-X group, and 4 chromosomes in the 21-22-Y group They were therefore interpreted as *having an XO sex-chromosomes constitution*

Thus in all the five patients a Y-chromosome was found somewhere in the tissues (Fig 3)

If it may be supposed that the chromosome complement in the gonad from which the tumour arose is the same as in the examined tissues (Leucocytes from peripheral blood and/or cells from skin) it is strongly suggestive, that the gonadal anlage should have developed into a testis This is so because it is generally believed that the presence of testicular tissue should be connected with the presence of the Y-chromosome

It is true, that one can find several cases in the literature in which presence of testicular tissue is claimed to be found without a Y-chromosome In a review of such cases by *Atkins & Engel* (1) only two pa-

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14 *Teter J & Tarlowski R* Tumors of the gonads in cases of gonadal dysgenesis and male pseud hermafroditism Am J Obst Gynec *79* 321 1960
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Acta path et microbiol scandinav 61, 551-557, 1964

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THE NATURE OF SHEEP CELL AGGLUTININS AND OX CELL HAEMOLYSINS RELATED TO MONONUCLEOSIS INFECTIOSA

By

ÖRJAN STRANNEGÅRD and LARS A HANSON

Received 11 xii 63

In 1932 *Paul & Bunnel* showed the presence of high titres of sheep erythrocyte agglutinating antibodies in sera from patients suffering

be absorbed by sheep erythrocytes to any measurable extent and concluded that it seemed probable that the haemolysins and agglutinins were different antibodies Recent studies by *Strannegård & Lycke* (in press), however, gave results suggesting that the sheep cell agglutinins could also function as ox cell haemolysins but nevertheless ox cell haemolysins existed which could not agglutinate sheep cells

The electrophoretic mobility of sheep cell agglutinins was investigated by *Faure et al* (1955) who found that these antibodies migrated with the β_1-, β_2- and fast-moving , globulins, that is they had the same migration rate as the isoagglutinins anti A and anti-B

Frenger (1957), using an agglutination electrophoresis in paper method investigated ten sera from M I patients and found sheep cell agglutinating activity in the γ-globulin region One serum with high sheep cell agglutinin titre showed, in addition, some agglutination in the β-region

Ultracentrifugation studies by *Kunkel* (1960) and investigations on the action of sulphydryl compounds on agglutinins by *Grubb & Swahn* (1958) indicated that sheep cell agglutinins in sera from patients with M I were high molecular weight antibodies

The aim of the present investigation was to compare the sheep cell agglutinins and the ox cell haemolysins which appear in sera from patients with M I as to their antigenicity, electrophoretic mobility and sulphydryl sensitivity

13 *Teter J Węgrowicz G Marzinek K Przezdziecki Z & Groniowski J* Tumeur a cellules germinales mixtes hormonalement active (gonadoblastoma) La Revue Francaise d Endocrinologie Clinique 3 421, 1962
14 *Teter, J & Tarlowski, R* Tumors of the gonads in cases of gonadal dysgenesis and male pseudohermafroditism Am J Obst Gynec 79 321 1960
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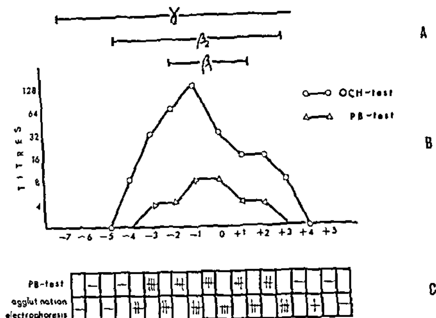


Fig 1

The electrophoretic mobilities of ox cell haemolysins and sheep cell agglutinins
The localization of ox cell haemolysins and sheep cell agglutinins after electrophoretic separation of M I sera at 5 V cm for 70 min was determined by means of two methoc
the elu
(C) Ic
phores

RESULTS

When the agglutination electrophoresis technique of *Hanson et al* was used, the sheep cell agglutinins were found to have an electrophoretic mobility similar to human serum β_2-globulins (γ_1-globulins) (Fig 1C) Agglutination occurred in the basins when the separated serum had been absorbed with guinea pig kidney, but not when it had been absorbed with boiled ox erythrocytes

With the technique of *Faure et al* the eluates of 6 separated sera were tested simultaneously for the presence of sheep cell agglutinins as well as ox cell haemolysins When the eluates were tested undiluted, different sites of maximum haemolysis were observed in different sera, but all eluates giving some haemolysis represented areas within the region of β_2-globulins The eluates of 4 sera were titrated and the maximum titres were in these cases observed in exactly the same region *Fig 1B* shows the results of an electrophoretic experiment with titration of the haemolytic and agglutinating activity of the eluates The results of agglutination electrophoresis are given in the lower part

MATERIAL AND METHODS

Antisera M I sera were selected from samples sent to the Virological Laboratory for diagnostic purposes

Anti human globulin sera were obtained from Behringswerke (Mahrburg/Lahn Germany) The anti- γ- and anti β_{2A} globulin sera were produced in rabbits whereas the anti β_{2M} globulin was obtained from horse The anti β_{2A} globulin serum was absorbed with γ globulin An anti I serum was obtained by absorbing anti γ globulin serum with the S fragment of γ globulin (S and F stand for slow and fast moving fragments of γ globulin produced by cleavage with papain)

Paul Bunnell (P B) test To 0 5 ml of each serum dilution was added 0 5 ml of a 1 per cent suspension of washed sheep erythrocytes and 1 0 ml of veronal buffer pH 7 0 The tubes were left overnight in the refrigerator and then read Readings were again performed after incubation for 2 hours at 37° C

O C H test To 0 5 ml of the serum dilution was added 0 5 ml of a 2 per cent suspension of ox erythrocytes 0 5 ml of 15 per cent guinea pig serum and 0 5 ml of veronal buffer pH 7 0 Readings were made after incubation in a waterbath at 37° C for 15 min Only inactivated sera were used for the O C H as well as the P B tests

Absorption tests Routine absorption tests of the patient sera with heated guinea pig kidney emulsion and ox erythrocytes were made using 0 25 ml of serum and 1 25 ml of the absorbents

In addition some of the test sera were absorbed with antisera to β_{2A} β_{2M} or γ globulins These antisera had previously been tested immunoelectrophoretically for their specificity to human serum proteins For the absorption experiments equal volumes of M I serum and antiserum were allowed to react for one hour at 37° C and after centrifugation the supernatant was tested with the P B and O C H tests The latter absorptions were controlled by means of double diffusion in gel experiments

2 Mercaptoethanol tests The effect of 2 mercaptoethanol (2 ME) on agglutinin and haemolysin activity was tested according to *LoSpalluto et al* (1962) Isotonic saline phosphate buffer, pH 7 2 containing 0 05 M 2 ME was used The P B and O C H tests were made as described before but instead of veronal buffer the 2 ME buffer

The experiments were performed in 1
the electrophoretic separation A 0 05 M
separation was made at 5V/cm for 60
gitudinal basins were cut in the agar a
permit development of the precipitation spectra

Agar gel electrophoresis for determination of antibody electromobility was performed according to the principles reported by *Faure et al* (1955) After separation agar pieces 3 × 15 mm with the short side parallel to the direction of the electric field were cut out of the agar The agar pieces were then eluted in small tubes each containing 0 25 ml of 0 85 per cent NaCl After at least one day in the refrigerator the eluates were tested for the presence of sheep cell agglutinins and ox cell haemolysins The localization of the cut agar pieces compared to the electrophoretic fractions was determined by developing the immunoelectrophoretic spectrum with the aid of longitudinally cut immune serum basins containing anti human plasma serum from sheep

In addition to the technique described above the method of *Hanson Raunio & Wadsworth* (1960) for localization of antibody activity after electrophoresis was used After completion of electrophoretic separation a matrix (78 × 15 mm) in which basins 3 mm in diameter had been formed was placed on top of the agar over the separated material The basins were about 1/3 filled with saline and the plate was then kept at 37° C for 30 min To determine agglutinating activity the basins were then filled with a 2 per cent suspension of sheep erythrocytes After 1 hour the material in each basin was mixed with a capillary pipette removed and finally microscopically examined for agglutination The localization of the serum fractions after the electrophoretic separation was determined by [illegible] comparison with the immunoelectrophoretic spectrum developed on the same plate

This latter technique was not easily applicable for the localization of ox blood haemolysins

DISCUSSION

The nature of antibodies formed in response to antigenic substances is dependent upon the character of the antigen and also upon the species of animal producing the antibodies. Ultracentrifugation studies have shown that the major part of antibodies are globulins with a sedimentation rate of 7 S or 19 S In man most antibodies are of the 7 S type Certain antibodies, however, occur primarily as 19 S molecules [illegible] agglutinating antibodies against bacteria, Wasser- [illegible]

the Forssman antigen are known to be electrophoretically [illegible] globulins (*Paic* 1939)

The results of the present investigation suggested that the heterophilic ox cell haemolysins and sheep cell agglutinins occurring in sera from patients with M I were β_{2M}-globulins (19 S γ-globulins) All the sera investigated showed antibody activity in the same electrophoretic region and the antibodies appeared to be susceptible to degradation by means of 2 mercaptoethanol *Kunkel et al* (1960) demonstrated by density gradient ultracentrifugation of sera from three patients with M I that the sheep cell agglutinating activity was confined to the 19 S fraction and *Faure et al* (1955) found this activity in the fast moving γ-globulin fraction *Grubb & Swahn* (1958) investigated the action of sulphydryl compounds on different agglutinins and found that heterophilic sheep cell agglutinins from patients with M I were destroyed by these compounds The present results were confirmed by the findings of *Strannegård & Lycke* (in press) which indicated that precipitating antibodies to ox cell antigens found in sera from patients with M I were β_{2M}-globulins Our findings are also consistent with those of *Pike & Schulze* (1960), who demonstrated that M I antibodies could not sensitize sheep cells for agglutination with rheumatoid factor The explanation for this should be that the M I antibodies were 19 S γ-globulins and rheumatid factor was specific for 7 S γ-globulin and therefore gave no reaction with the cells coated with 19 S antibodies

Studies by *Taliaferro* (1957) and *Stelos & Talmage* (1957) showed that rabbits produced two types of antibodies when immunized with sheep erythrocyte stroma The first type had a high molecular weight, was strongly haemolytic and reached peak titres early after vaccination The second type had weak haemolytic activity, attained peak titres later than the first type and had a molecular weight of about 165,000 *LoSpalluto et al* showed that after initial immunization of human subjects with typhoid paratyphoid antigens high molecular weight (19 S) antibodies were produced After some time there was a gradual change in antibodies from 19 S to predominantly 7 S type This was, however, not true for the typhoid O agglutinins which were found in the 19 S fraction irrespective of the time elapsed since the initial immunization

The heterophilic antibodies appearing in sera from patients with M I

of the figure (Fig 1C) The results indicate that the antibodies tested have the mobility of β_2-globulins (Fig 1A)

The absorption experiments with specific immunoglobulin antisera showed that anti-γ, anti-F and anti-β_{2A} sera in no case lowered the O C H or P -B titres, whereas anti-β_{2M} serum evidently inhibited the ox cell haemolysins and sheep cell agglutinins (Table 1)

TABLE 1

Effect of Absorption of M I Serum with Specific Anti-Sera to the Immune Globulins

	Titres after absorption with			
	Normal rabbit or horse serum	Anti β_{2A}	Anti β_{2M}	Anti F
P -B test	128	128	<32	128
O C H test	1024	1024	<32	1024

P B and O C H titres of a serum from a patient with suspected M I after absorption with normal rabbit serum, normal horse serum, and antihuman globulin sera The anti β_{2M} globulin serum was from a horse whereas the anti β_{2A} and anti γ globulin sera were obtained from immunized rabbits Equal volumes of test serum and antiserum were allowed to react for one hour at 37° C and after centrifugation the supernatant was titrated and tested for presence of sheep cell agglutinins and ox cell haemolysins

Treatment of sera with 2-ME resulted in marked decrease of the haemolysing and agglutinating activities Ox cell haemolysins could in no case be demonstrated in the lowest dilution tested after treatment Sheep cell agglutinins, however, could be demonstrated in some sera in low titres when readings of the P B tests were made after keeping the tubes in the refrigerator overnight After additional incubation for 2 hours at 37° C no agglutination could be demonstrated Antibodies in sera obtained at different phases of the disease were evidently equally sensitive to 2-ME treatment (Table 2)

TABLE 2

Effect of Treatment with 2 Mercaptoethanol

Patient	Serum no	Time since onset of disease in days	Titres			Titres after treatment with 2 ME		
			P B cold	P B warm	O C H	P B cold	P B warm	O C H
AKJ	1103	24	512	256	8192	16	<4	<4
AKJ	1373	57	32	16	128	<4	<4	<4
LGJ	500	8	512	256	16384	32	<4	<4
LGJ	994	69	64	16	512	8	<4	<4
LGJ	1479	129	16	8	64	<4	<4	<4
LJ	1982	12	256	128	4096	16	<4	<4
LJ	2346	52	256	32	1024	16	<4	<4

Seven sera from three patients with M I obtained at different times since the onset of the disease were tested for occurrence of sheep cell agglutinins and ox cell haemolysins before and after treatment with 0 05 M 2 mercaptoethanol All sera were absorbed with guinea pig kidney before the tests were performed

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usually disappear from the blood within a few months Most of the sera tested in the present investigation were obtained rather early after the onset of the disease and the finding that the antibodies demonstrated appeared to be β_{2M}-globulins (19 S globulins) was thus similar to the results of previous investigators as regards some typhoid-paratyphoid antibodies and sheep cell agglutinins in immunized rabbits The possibility of the existence of M I antibodies of the 7 S variety cannot be completely excluded The fact, however, that even sera from patients obtained up to 4 months after the onset of the disease did not show 2-mercaptoethanol resistant antibody activities makes it probable that these antibodies do not change character with time

SUMMARY

The sheep cell agglutinins and ox cell haemolysins occurring in sera from patients with mononucleosis infectiosa were investigated by means of treatment with a sulphydryl compound, by means of electrophoresis and by inhibition by specific immune sera to the immune globulins Both types of antibodies appeared to be β_{2M}-globulins irrespective of the time elapsed since the onset of the disease

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shown, too that the inagglutinability of heat stable antigens in live nutrient agar cultures observed at times by Oeding (38), could be abolished by using live mannitol salt agar cultures as antigens

It appears clearly from these investigations that the antigenic structure of *Staph aureus* is very complex, and the agglutinogens numerous This complexity is also seen on studying extracts of staphylococcal cells by double diffusion techniques (5, 6, 10, 31, 40, 55)

It was felt, therefore, that investigations on the antigenic structure of pathogenic staphylococci had, for the present, to be limited to a thorough study of selected "types" or groups of related strains For this purpose *Staph aureus* strains within the 80 81 complex were chosen for further studies

The phage 80 was described by *Rountree & Freeman* (52) in 1955 About the same period, *Bynoe et al* (3) reported from Canada another phage 81 The relationship between staphylococcal strains lysed by

[illegible]

demonstrated by several investigators (36) The designation 80 81 complex used by *Nahmias et al* (36) for these strains seems therefore reasonable and has been adopted in this study

Pathogenic staphylococci within the 80 81 complex have been recognized as particularly epidemic strains (11, 58) Knowledge of the antigenic structure of these strains was found to be of interest, not only in itself but also for epidemiological work and for further research in the field of infection and immunology

The present paper deals with the agglutinogens found in these strains A preliminary note was published in 1962 (27)

MATERIALS AND METHODS

Strains Strain 263 has been used throughout this study The strain was isolated from a carbuncle during a hospital epidemic It has the phage pattern 80 81/82/KS6 The strain ferments mannitol and gives positive reactions for bound and free coagulase It produces hyaluronidase fibrinolysine esterase and phosphatase and is egg yolk pos tive (12 Strain 263 is virulent for mice as 3 × 10^8 microbes injected intramuscularly produced abscesses leading to death in some animals

In addition the following stra ns were used

[illegible] owan s type I S11 (NCTC 8530) (NCTC 8532) and the additional Haukenes (19) [illegible] pe 4 (NCTC 6131) type 5 (NCTC [illegible] ype 8 (NCTC 6136) type 9 (NCTC [illegible] e 11 (NCTC 8723) type 12 (NCTC

[illegible] ford strain (Public [illegible] er strain A and the [illegible] Philadelphia) The [illegible]) and the two colonial variants Smith diffuse" and Smith compact (29) (Furn shed by M Glenn

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STUDIES ON THE ANTIGENIC STRUCTURE OF THE 80/81 COMPLEX OF STAPHYLOCOCCUS AUREUS

1 *Agglutinogens*

By

TOR HOFSTAD

Received 31 xii 63

Some attempts have been made to classify *Staphylococcus aureus* by serological methods *Hine* (1922), *Julianelle* (1922), *Yonemura* (1936) and *Cowan* (1939) showed that pathogenic staphylococci could be subdivided by means of agglutination reactions

Cowan, using slide agglutination of bacterial cells in non-absorbed and absorbed rabbit immune sera, recognized three main serotypes in *Staph aureus* Boiled bacteria were used for the production of immune sera, for absorption and for agglutination By means of the same technique the number of serotypes was extended to nine by *Christie & Keogh* (4), and to 13 by *Hobbs* (26) This line of work was taken up by *Mercier, Pillet & Chabanier* (35), and continued by *Pillet et al* (45, 46, 47, 48)

Another antigenic scheme for *Staph aureus* was worked out by *Oeding* in 1952 (37) In order to obtain a more complete picture of the antigens in pathogenic staphylococci he used formalin-killed bacteria in the production of immune sera, and live bacteria, grown on nutrient agar, were used for both absorption and slide agglutination Selected strains were studied by cross absorptions, on the basis of which ten antigens, named by letters from *a* to *k*, were recorded Factor sera were made for seven of these antigens (Factor *a,b,c,e h,i* and *k* sera) *Oeding's* results were confirmed by *Grun* (13), who also described a new antigen *l* (14) Four new antigens, *m,n* and *o p*, were later described by *Haukenes & Oeding* (24) and by *Grun* (15), respectively

Still another line of work, based on the type strains used by *Cowan*, *Christie & Keogh* and *Hobbs*, and on *Oeding*'s technique, has been taken up by *Torres Pereira* (54)

Oeding's factor *a,b,c h,i* and *k* sera have recently been re-examined by *Haukenes* (17, 18, 19 20, 21), who found that each of them contained agglutinins against two or more antigens *Haukenes* has also found other new antigens in *Oeding's* and *Cowan*'s type strains (22) He has

EXPERIMENTAL PROCEDURE AND RESULTS

Typing of Strain 263 in Known Factor Sera.

Strain 263 was first typed in factor a,b,c,e,h,i,k,m and n sera, which revealed the antigenic formula $abc(k)m$.

The strain has later been re-examined by agglutination and absorption in the new factor $a_1,a_2,c_1,h_1,h_2,k_1k_2$, Cowan-factors-1-2, 670-1, 5687-1 and 6376-1 sera The a,b and c sera have been absorbed with strain 263 alone and in combination with known type strains On the basis of these experiments strain 263 has been found to possess the following agglutinogens. $a_2,(k_2),m$, Cowan-factor-1 and 5687-1, in addition to agglutinogens shared by all or most *Staph aureus* strains (referred to as group antigens hereafter) and other agglutinogens to be described below The k_2 antigen was revealed only by absorption

Agglutinating Antibodies in Immune Serum 263

In a pilot experiment two rabbits were immunized with strain 263 *Both immune sera were absorbed with each of the type strains (ie* 1503, 2253, 28, 365, 3647, F21, 17A, 3189, 2095, Wood 46, Cowan I, Cowan II, Cowan III) and with combinations of these strains, and then examined for residual agglutinins The absorption experiments disclosed at least two previously unknown antigens in strain 263 Table 1 *shows the* distribution among the type strains of the two new antigens Absorption of immune serum 263 with nutrient agar cultures of strain 3647 and F21, which removes group antigens and a_2 and m agglutinins, *left* agglutinating antibodies to strains 17A, Cowan I, Cowan II and 263 Additional absorptions with strain 365 or strain 1503, and with mannitol-salt agar cultures of the new type strain 670, which would have removed any undetected k_2, Cowan-factor-2 and 5687-1 agglutinins (23) did not weaken the positive agglutinations Agglutinating antibodies to strains Cowan I and 263 are still left after additional absorption with *strains 17A or* Cowan II Absorption with strains Cowan I or 263 exhausts the immune serum

TABLE 1

Distribution among the Type Strains of the 263 1 and 263-2 Antigens

Type strains	Immune serum 263 absorbed with			
	3647 F21	3647 F21 17A	3647 F21 Cowan II	3647 F21 Cowan I
17A	+	—	—	—
Cowan I	+++	+++	+++	—
Cowan II	++	—	—	—
263	+++	+++	+++	—
Other 10 strains	—	—	—	—

+, ++, +++ Degree of agglutination — No agglutination

Antigen Live 18 hours nutrient agar slant cultures

Koenig Nashville) The strain 137, isolated in this laboratory, and a few other strains mentioned in the text

Six hundred and fifty eight coagulase positive strains from routine specimens isolated in 1959 and 1960

The strains have been stored in the freeze dried state, or maintained in nutrient agar in screw cap bottles

Factor sera All factor sera have been prepared from heat inactivated (½ h 56° C) immune sera diluted 1 10 in saline

Factor *a* *b* c, *e*, *i* and *k* sera were prepared as proposed by *Haukenes* & *Oeding* (24), factor a_4 c_1 h_2 k_1k_- *m* Cowan factors 1 2, 670 1, 5687 1 and 6376 1 sera as proposed by *Haukenes* (17, 19, 20, 21, 22)

The other factor sera were prepared as follows

Factor *n* serum Immune serum 1503 was absorbed with nutrient agar cultures of strains 2095 Cowan I and 137, because serum 1503 gave a stronger factor *n* serum than serum Cowan III (24) Strain 1503 has the antigenic formula $a_1b_\circ ac_1emn$ Cowan factors 2 4 (19 22, 24) Strain 2095 and Cowan I absorb agglutinins to $a_1b_-ac_1$ and *m* Cowan factors 2 4, respectively, (in addition to agglutinins shared by most or all *Staph aureus* strains) Strain 137 has a strong *e* antigen, but no *n* antigen (personal investigations) and will consequently absorb *e* agglutinins Strain 137 is used for this purpose only

Factor *a* serum was first prepared from immune serum 17A (20) This factor serum was, however, rather weak A factor a_1a_3 serum was therefore prepared by absorbing immune serum 2095 with nutrient agar cultures of strains F21 3189 and Cowan I Strain 2095 has the antigenic formula $a_1a_2a_3a_4a_5b_1b$ b_3ac_1 (19) Strains F21, 3189 and Cowan I absorb agglutinins to $b_1b_2ac_1$ a_3b_3 and a_- respectively a_1 agglutinins were not found in the immune serum used

Factor h_1 serum Immune serum 17A was absorbed with nutrient agar cultures of strain 3647 and mannitol salt agar cultures of strain Cowan I Strain 17A has the antigenic formula $a_-a_5b_3h_1h_2$ Cowan factors 2 4 5687 1 (19 20 22), $a_2a_5b_3$ and h_2 Cowan factors 2 4 5687-1 agglutinins are absorbed by 3647 and Cowan I micr bes respectively Mannitol salt agar cultures were found superior to nutrient agar cultures in absorbing agglutinins to the heat stable h_2 antigen

Merthiolate was added for preservation of the factor sera which were used freshly prepared or after storage at 4° C for up to one year Absorbed rabbit sera diluted 1 10 in saline were used as negative controls

Immunization Rabbit immune sera were produced by intravenous injections of formalin killed microbes as described by *Oeding* (39) A few rabbits were also immunized with live microbes starting with 10^{6} or 10 organisms in 0 1 ml saline All rabbits were tested for normal agglutinins before starting immunization and only rabbits giving titres of 1 10 or less to the immunizing strain have been used

Agglutination was performed on slides using live fresh 18 hours nutrient agar slant cultures or in some instances mannitol salt agar cultures grown at 37° C as antigen (20)

All *antibody absorptions* were performed with live microbes grown on nutrient agar and/or mannitol salt agar Plates were for practical purposes used instead of Roux bottles (The growth on one plate 14 cm diameter corresponds roughly to three quarter of the growth in one Roux bottle)

The factor sera were with some exceptions prepared by absorption with standard doses of bacteria (33) As unspecific absorption seemed to be negligible most other antibody absorptions were carried out with excess antigen In most instances 1 ml of diluted serum was absorbed with the growth from one nutrient agar plate and in some instances one mannitol salt agar plate in addition The absorptions were performed at 37° C for two hours with repeated stirring and the specimens were then left in the refrigerator overnight before centrifugation

Phage typing The phages 81 82 (8) and KS6 (57) which were all placed in phage group I have been used in additi n to the basic set (30) The phage typing was otherwise performed as described earlier (28)

Trypsin digestion Live microbes were incubated at 37° C for two hours with solutions of freshly prepared crystalline trypsin (Trypure Novo in 1 M trisbuffer pH 7 8 Similar solutions inactivated by heating to 100° C for ten minutes were included as controls

TABLE 2

Agglutinability of all Type Strains in Factor 263 1 and 263 2 Sera

Type strains	263-1 serum	263-2 serum
17A	—	+*
Cowan I	+++	+*
Cowan II	—	++
263	+++	+
5687	—	+
Other 14 strains	—	—

Code See Table 1
* Sometimes blocked in agar cultures.

Factor 263 1 serum Immune serum 263 absorbed with strains 3647, F21, 17A
Factor 263 2 serum Immune serum Cowan II absorbed with strains 3647,670

Factor 263-1 and 263-2 sera have both been absorbed with nutrient agar and mannitol-salt agar cultures of the non-agglutinating type strains and with the additional type strains 1015, 670, 5687, 830 and 6376 Strain 5687 exhausted 263-2 serum, otherwise the absorptions did not alter the agglutinating patterns shown in Table 1

The agglutinability of all 19 type strains in factor 263-1 and 263-2 sera is shown in Table 2 together with the proposed procedure for preparation of the two factor sera

Factor 263-1 and 263-2 sera have been absorbed with 15 independent, agglutinating staphylococcal strains All strains exhausted the factor sera This, in addition to the results obtained by absorptions of immune sera 263 and Cowan II carried out for other purposes, suggest that they are both monovalent factor sera

Sensitivity of the 263-1 and 263 2 Antigens to Heat and Trypsin

Eighteen hours nutrient agar cultures of strain 263, harvested in minimum saline or by scraping with a glass rod, were heated to 100° C in a water bath After heating for one hour the microbes were inagglutinable in factor 263-1 and 263-2 sera, but some agglutinin-binding property was still found Autoclaving at 120° C for two hours destroyed the agglutinin binding property of the two antigens The agglutinability in factor 263-1 and 263-2 sera seemed unimpaired after heating at 60° C for one hour and was only slightly reduced after five minutes' boiling

Both antigens were susceptible to digestion with trypsin The agglutinability and the agglutinin binding property of 1 mg dry weight of microbes were destroyed by 0 2 mg (263 1) and 0 4 mg (263 2) of crystalline trypsin

263 1 Agglutinins in Immune Sera to other Strains within the 80 81 Complex

Eight rabbits were immunized with eight other independent staphylococcal strains belonging to the 80 81 complex Three of the strains had

The agglutinating patterns shown in Table 1 were also found when mannitol-salt agar cultures were used as antigen

The agglutinogen shared by strain Cowan I and strain 263 has been provisionally named 263-1 The other new antigen, shared by strains 17A, Cowan I, Cowan II and 263, has provisionally been named 263-2 Weak *m* agglutinins were found in both sera, and also weak agglutinating antibodies to an antigen shared by strains 3647, 17A, 2095, Cowan III and 263 They might have been a_3 agglutinins However, they were absorbed by strain Cowan I, which suggest the presence of another new antigen (263-3).

In a subsequent experiment five rabbits were immunized with strain 263 after the strain had been passed through mice All immune sera yielded strong agglutinins to the 263-1 antigen Agglutinating antibodies to 263-2 were found in two of the sera k_2 agglutinins were found in neither of the immune sera

In another experiment three rabbits were immunized with live 263 organisms One rabbit was immunized with fresh 18 hours nutrient agar cultures, the other two were immunized with freeze dried organisms originating from five and eight hours nutrient agar cultures from an infected mouse and a furuncle (the author's), respectively Absorption experiments did not disclose any new antigens, and the immune sera were readily exhausted by absorption with formalin-killed 263 organisms 263-1 agglutinins were found in two immune sera

Preparation of Factor 263-1 and 263-2 Sera

A potent factor 263-1 serum was prepared by absorbing immune serum 263 with nutrient agar cultures of strains 3647, F21 and 17A, which remove all other known agglutinins, except k_2 agglutinins (cf Table 1) k_2 agglutinins can, if present, be removed by additional absorption with strain 365 A strong and immediate reaction is given by all staphylococcal strains agglutinating in this factor serum Nutrient agar slant cultures and mannitol-salt agar cultures can both be used as antigen

Factor 263-2 serum can be prepared from immune serum 17A, or better, from immune serum Cowan II by absorbing the latter with nutrient agar cultures of strains 3647 and 670 Strain Cowan II has the antigenic formula b_3h_2 Cowan-factors-2-3-4 5687-1 263-2 (19, 20, 22) Strain 3647 absorbs group antigens and strain 670 absorbs all specific agglutinins except Cowan-factor-3 and 5687-1 agglutinins Cowan-factor-3 and, probably, 5687-1 agglutinins were not found in the immune serum used Nutrient agar slant cultures and mannitol-salt agar cultures can both be used as antigen Recent investigations suggest, however, that mannitol-salt agar cultures are to be preferred to nutrient agar cultures, as antigen 263-2 sometimes has been found to be very weak or blocked in agar cultures of strains 17A and Cowan I

examined in factor 263-2 serum Agglutinating strains were found within phage groups I, II and the mixed group, and among the non-typable strains in addition.

Staph aureus Oxford was negative in factor 263-1 serum, but agglutinated in factor 263-2 serum The serological type strains 4 to 13 were negative in both sera So also were the Smith mother strain and the two colonial variants, and strain 209-P

Strain D-86 agglutinated in factor 263-1 and 263 2 sera *Grun's* other type strains A4, D-124 and D-142 were negative in both factor sera

Krapral & I i s 18Z strains all agglutinated in factor 263-1 and 263-2 sera

TABLE 3

Agglutination of 658 Staph aureus Strains in Factor 263 1 Serum

No of strains	Phage group	Lysis at	Agglutination	No agglutination
245	I	RTD	245	
46		RTD × 1 000	8	38
33	II	RTD RTD × 1 000		33
138	III	RTD RTD × 1 000		138
1	IV	RTD		1
14	Misc (187)	RTD RTD × 1 000		14
123	Mixed	RTD RTD × 1 000	6*	117
58	NT		10	48
658			269	389

RTD = Routine Test Dilution
NT = Not typable
* 29/3A/3B/3C/7 54 83 in RTD × 1 000

Subtyping of 263-1 Positive' Strains

Fifty staphylococcal strains which all agglutinated in factor 263-1 serum have also been typed in factor $a,b,c,i,k,a_1,a_1a_2,c_1,h_1,h_2,m,n$ and 263 2 sera The serological typing, with the exception of typing in a_1,c_1 and h_2 sera was carried out with fresh 18 hours nutrient agar slant cultures as antigen Eighteen hours cultures were used for practical reasons as identical results had been obtained with five and 18 hours cultures with a single exception (a_1 serum), in preliminary experiments The typing *in a_1 serum was performed with five hours nutrient* agar slant cultures and 18 hours mannitol salt agar cultures were used for typing in c_1 and h_2 sera The other new factor sera described by *Haukenes* were at that time, either not available or too weak for practical use This was also the case with our *c* serum

Nearly all strains agglutinated in the polyvalent *a b* and *c* sera Positive reactions were found in the monovalent h_1 h_2,m and 263-2 sera, and in the divalent a_1a_2 serum (but not in a_1 serum) (Table 4) A few *strains agglutinated in k* serum, but this factor serum was rather weak and could be partly absorbed by some non-agglutinating strains

Several antigenic formulas were found among strains within the

been isolated in foreign laboratories (Stockholm, Malmo, Copenhagen) The others were isolated in this laboratory from specimens originating from different parts of Norway

Agglutinating antibodies to antigen 263-1 were found in all immune sera, but only three immune sera yielded a factor 263-1 serum strong enough for practical use Agglutinins to unknown antigens could not be found

These strains and strain 263 were able to exhaust each other's immune sera in cross-absorption experiments. Subsequent typing in several factor sera showed, however, that there was a slight difference between three of the strains and strain 263.

Agglutinating antibodies to antigen 263-1 have not been found in immune serum Cowan I

Agglutinating Antibodies in Immune Sera to other Staphylococcal Strains within Phage Group I.

In another experiment rabbits were immunized with ten phage group I strains with other phage patterns than those found within the 80 81 complex, and with two non-typable strains which both agglutinated in factor 263-1 serum. The immune sera were analysed in the same way as immune sera 263.

Strong agglutinins to unknown antigens were found in immune sera to three of these strains (phage patterns Non-typable in the Routine Test Dilution (RTD) 52 w, 52/52A w and 79 v w in RTD × 1000) The three strains, which did not agglutinate in factor 263-1 and 263-2 sera, showed on subsequent typing in other factor sera antigenic formulas unlike strains within the 80/81 complex

The most outstanding finding in the immune sera was the presence of strong h_2 agglutinins Weak 263-1 and 263-2 agglutinins were found in some sera

The Distribution of the 263-1 and 263-2 Antigens among Coagulase Positive Staphylococci

Six hundred and fifty-eight coagulase positive strains of staphylococci have been examined in factor 263-1 serum (Table 3) All strains within phage group I which are lysed by diluted phages agglutinate in factor 263-1 serum, and so do a few strains which are lysed by concentrated phages only The six strains from the mixed group which gave a positive reaction, and the ten positive non typable strains have been shown to be similar to strains within the 80 81 complex with respect to other antigens as well None of the strains within phage groups II, III and IV, and strains lysed by the phage 187, agglutinated in factor 263-1 serum

One hundred and fifty staphylococcal strains, representing different phage patterns within all phage groups and non-typable strains, were

made direct comparison of these two antigens impossible The agglutinating patterns of the type strains in 263-2 and 5687-1 (22) sera suggest that these two factor sera may be identical, in spite of the discrepancies found after absorption with mannitol salt agar cultures of strain 670

The recognition of 263-1 and 263-2 has no consequences for the preparation of the other factor sera

Besides the regular occurrence of 263-1 agglutinins in immune sera to *Staph aureus* strains within the 80 81 complex, the outstanding finding in this study was the nearly complete correlation between phage group I and agglutinability in 263-1 serum (cf Table 3) A broad correlation between serotypes and phage groups of *Staph aureus* has been found earlier by other investigators using polyvalent sera (16, 26, 42, 43, 44, 56) This study indicates, however, that the new antigen 263-1 can be regarded as a type agglutinogen characteristic of staphylococcal strains within phage group I, i e the 80 81 complex and a few other related strains

A practical outcome of the above stated correlation is that typing in factor 263 1 serum can be used for screening before phage typing when searching for such strains

Typing in other factor sera showed that "263-1 positive" strains, although closely related serologically, can be subtyped

The lack of correlation between certain agglutinogens and lysis by particular phages, and the finding of different phage patterns among staphylococcal strains with simalar antigenic formulas (cf Table 4), are in keeping with the changes in phage patterns of strains within the 80 81 complex found after lysogenization (1, 2, 7, 50, 51) The different antigenic formulas among strains with similar phage patterns are comparable to the findings of subtypes within apparently identical type 80 81 strains (34, 53)

263-1 has earlier been named 'a group specific antigen" (27) Based on present knowledge of the antigenic make up of *Staph aureus*, the author s opinion is that the designation "group antigen" is best reserved for the antigens shared by all or most coagulase positive staphylococci

The new antigens described in this paper have, as the whole terminology has to be revised, been named provisionally according to the particular strain first examined Future terminology covering all agglutinogens in *Staph aureus* may most conveniently be based on numerals

SUMMARY

A thorough study, based on *Oeding's* technique of the agglutinogens in strains within the 80 81 complex of *Staphylococcus aureus*, is reported

Ten rabbits were immunized with the epidemic phage type 80 81/82/ KS6 strain 263 eight rabbits with other strains within the 80 81 complex, and ten rabbits with other phage group I strains All immune sera

80/81 complex Similar antigenic formulas were found among strains with differing phage patterns, and, on the other hand, strains with similar phage patterns differed as to their antigenic formulas No difference in agglutinating patterns were found between strains within the 80/81 complex and the few "263-1 positive" strains not included in this complex.

TABLE 4

Subtyping of 50 "263-1 Positive" Staph aureus Strains

No of strains	Antigenic formula	Phage pattern		
		80 81 complex	Other gr I patterns	Mixed NT
17	263-1 263-2 a_5 m	15		2
15	263-1 263-2 a_5 h_2 m	11	2	2
5	263-1 263 2 h_2 m	4	1	
2	263-1 263 2 m	2		
2	263-1 a_5 h_2 m	2		
2	263-1 a_5 m	2		
2	263 1 263-2 a_5 h_1 h_2 m	1	1	
2	263 1 263-2 h_1 h_2 m		1	1
1	263 1 263-2 a_5	1		
1	263-1 263-2 a_5 h_1	1		
1	263-1 h_2 m	1		
50		40	5	5

DISCUSSION

The present investigation has revealed two new *Staphylococcus aureus* antigens within the 80/81 complex Factor sera have been prepared to both of them One of these antigens, antigen 263-1, is undoubtedly a strong, major agglutinogen in strains within this complex Antibodies to this antigen have been found in all immune sera to the epidemic strain 263, and in immune sera to some other strains within the 80/81 complex

The other antigen, 263-2, is a relatively weak agglutinogen in these strains Antibodies to this antigen have been found in few of the immune sera studied

The susceptibility to heat and trypsin suggest that 263-1 and 263 2, like most other agglutinogens in *Staph aureus* (41, 49), are proteins 263-1 and 263-2 are not identical to *Grun*'s *l,p* or *o* antigens The German strain D-86 which agglutinated in factor 263-1 and 263-2 sera, was used by *Grun* for absorption only (15) 263-1 agglutinins may be present in factor sera prepared from immune serum Cowan I by *Pillet et al* (35, 47), and *Torres Pereira* These investigators are most probably working with polyvalent sera

263-1 and 263-2 may be identical to *Haukenes*' Cowan-factor-1 and 5687-1 antigens respectively Neither Cowan-factor-1 nor 263-1 agglu- ... been found in the Cowan I immune sera studied, which has

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were absorbed with known type strains, and thereafter examined for any remaining agglutinins

The investigation revealed two previously unrecognized antigens, provisionally named 263-1 and 263-2, to which factor sera have been prepared 263-1 agglutinins were regularly found in immune sera to strains within the 80/81 complex

Serological typing in factor 263-1 serum of 658 coagulase positive staphylococcal strains showed nearly complete correlation to phage group I The new antigen 263-1 can, therefore, be regarded as a type agglutinogen characteristic of strains within the 80/81 complex and other strains related to this complex

Typing of "263-1 positive" strains in other factor sera revealed several antigenic formulas, although the strains were found to be serologically related No difference in agglutinating patterns were found between strains within the 80/81 complex and other "263-1 positive" strains not included in this complex Nor was any correlation found between certain agglutinogens and lysis by particular phages

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ÜBER DAS VORKOMMEN UND DIE BEDEUTUNG DER SALMONELLA-SUB-GENUS II-SPECIES

Von

F Kauffmann und A Petersen

Eingegangen 20 xii 63

In einer vorhergehenden Mitteilung von *F Kauffmann „Zur Serologie des Salmonella Sub-Genus II"* wurden die Antigen-Formeln der bis Ende 1962 bekannten 131 *Salmonella-species* des *sub-genus* II (nach dem originalen *Kauffmann-White Schema*) zusammengestellt In Ergänzung dieser Arbeit wird in der folgenden Tabelle auf die Herkunft dieser *species* eingegangen Gleichzeitig werden die vereinfachten Antigen-Formeln angegeben, da diese bisher noch nicht im Zusammenhange publiziert worden sind In einer Mitteilung von *F Kauffmann & R Rohde „Zur Vereinfachung der serologischen Salmonella Diagnose"* war vorgeschlagen worden, ab 1 1 1963 alle neuen *species* des *Salmonella sub genus* II nach dem vereinfachten K W Schema zu diagnostizieren Wenn also in Zukunft neue *species* des *sub-genus* II beschrieben werden, so bilden sie eine Ergänzung der vorliegenden Arbeit

TABELLE

Vorkommen der Sub Genus II Species
(mit vereinfachten Formeln)

Species	Vereinfachte Formel	Mensch			Tier			
		Lat	Ges	°	W	h	V	E
		Gruppe B						
S sofia	B b	+				+		
*S sofia var 27	B b						+	
*S makumira	B en l				+			
*S caledon	B G en	+						
*S bechuana	B G –							
S kilwa	B L en						+	
S nordenham	B z en	+						
*S durbanville	B z_{39} l	+						
		Gruppe C						
S calvinia	C a z_{42}	+						
*S bloemfontein	C b z_{42}					+		
*S heilbron	C L l						+	
*S tosamanga	C z l	+					+	

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(mit vereinfachten Formeln)

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Gruppe B								
S sofia	B b	+				+		
S sofia var 27	B b						+	
*S makumira	B en 1				+			
*S caledon	B G en	+						
S bechuana	B G						+	
S kilwa	B L en	+						
S nordenham	B z en	+						
S durbanville	B z_{39} 1	+						
Gruppe C								
*S calvinia	C a z_{42}	+				+		
S bloemfontein	C b z_{42}						+	
S heilbron	C L 1						+	
*S tosamanga	C z 1	+						

TABELLE

Species	Vereinfachte Formel	Mensch			Tier			
		Pat	Ges	?	W	K	N	A
*S bacongo	C z_{36} z_{42}					+		
*S gilbert	C z_{39} l						+	
*S sullivan	C z_{42} l						+	
*S tulear	C a z_{52}					+		
*S baragwanath	C G l	+						
*S germiston	C G e n	+						
Gruppe D								
*S mjimwema	D b e n	+						
*S blankenese	D b z_6						+	
S zuerich	D c z_{39}					+		
S lindrick	D e n l					+		
*S lindrick var 1,7	D e n l					+		
*S kuilsrivier	D G e n	+						
*S manica	D G z_{42}					+		
*S neasden	D G e n	+						
S hamburg	D G –	+	+				+	
*S dar es salaam	D l e n	+						
*S stellenbosch	D z l	+						
*S angola	D z z_6						+	
S hueningen	D z z_{39}					+		
*S canastel	D z_{29} l		+					
*S wynberg	D z_{39} l			+				
*S lundby	D b e n						+	
S haarlem	D z e n							+
Gruppe E								
S chudleigh	I e n l							+
S islington	I G						+	
*S fuhlsbuettel	I L z_6						+	
*S westpark	I l e n					+		
*S alexander	I z l	+						
S finchley	I z e n						+	
*S mpila	I z_{38} z_{42}					+		
S winchester	I z_{39} l				+			
*S parow	L G			+				
Gruppe F								
S montgomery	11 d(a) d e n		+					
*S grabouw	11 G z_{39}			+				
S lincoln	11 G e n			+				
*S huila	11 L e n						+	
S parera	11 z_4							+
Gruppe G								
*S limbe	13 G –			+				
S rotterdam	13 G l					+		
S clifton	13 z_{29} l					+		
*S goodwood	13 z_{29} e n	+						

TABELLE

Species	Vereinfachte Formel	Mensch Ent	Ges	*	Tier W	K	S	A
S acres	13 b $z_{4?}$	+						
S luanshya	13 G –			+	+			+
S gojenberg	13 G 1						+	
S katesgrove	13 G 1							
S worcester	13 G e n	+						
S narhshonim	13 z 1	+						
S stevenage	13 $z_{4?}$ 1			+				
Gruppe I								
S bellville	16 e n 1	+						
S m beni	16 G							
S merseyside	16 G 1			+				
S rowbarton	16 G					+		
S haddon	16 z_4	+						
S jacksonville	16 z_{29}				+			
S woodstock	16 $z_{4?}$ 1	+						
S elsiesrivier	16 z_4 1	+						
Gruppe J								
S hillbrow	17 b e n	+						
S verity	17 e n 1					+		
S bleadon	17 G e n					+		
Gruppe K								
S ze st	18 z_{10} z_6					+		
S beloha	18 z_{36}					+		
Gruppe L								
S s esterberg	21 z_4					+		
S gwaai	21 z_4				+			
S wandsbek	21 z_{10} z_6						+	
Gruppe M								
S kaltenhausen	28 b z_6				+		+	
S ceres	28 z z_{39}	+						
Gruppe P								
S carlet nville	38 d							
S foulpointe	38 G					+		
Gruppe Q								
S mondeor	39 L e,n			+				
Gruppe R								
S springs	40 a z_{39}	+						
S suarez	40 c e n					+		

TABELLE

Species	Vereinfachte Formel	Mensch			Tier		N	V
		Pat	Ges	?	W	K		
S alsterdorf	40 G –						+	
*S boksburg	40 G e n	+						
*S bulawayo	40 z l				+			
S sachsenwald	40 z_4 –				+			
S degania	40 z_4 –	+						
S bern	40 z_4 –					+		
S fandran	40 $z_{3,}$ e n					+		
S grunty	40 z_{39} l						+	
Gruppe S								
S lethe	41 G –					+		
S negev	41 z_{10} l	+						
S lichtenberg	41 z_{10} z_6					+		
Gruppe T								
S chinovum	42 b l						+	
S uphill	42 b e n					+		
S fremantle	42 G –	+						
*S portbech	42 L e n						+	
*S nairobi	42 r –				+			
S detroit	42 z l					+		
*S rand	42 z e n			+				
Gruppe U								
S houten	43 z_4 –					+		
S tuindorp	43 z_4					+		
S bunnik	43 z_{42} l				+	+		
Gruppe W								
S vrindaban	45 a e n				+			
*S ejeda	45 a z_{10}					+		
*S bremen	45 G e n						+	
*S windhoek	45 G l	+						
*S perinet	45 G e n					+		
*S klapmuts	45 z z_{39}					+		
Gruppe X								
S bilthoven	47 a					+		
S phoenix	47 b l	+						
*S quimbamba	47 d z_{39}						+	
*S chersina	47 z z					+		
Gruppe Y								
S hammonia	48 e n z_6					+		
*S sakaraha	48 k z_{39}					+		
*S ngozi	48 z_{10} l					+		

TABELLE

Species	Vereinfachte Formel	Mensch Pat	Mensch Ges	Mensch ?	Tier W	Tier K	N	A
Gruppe 50								
S krugersdorp	50 e n 1					+		
S wassenaar	50 G							
S greenside	50 z e n		+					
S bonaire	50 z_4 –				+			
S hooggraven	50 z_{10} z_6 z_{42}					+		
Gruppe 51								
S roggeveld	51 – 1					+		
Gruppe 52								
S lobatsi	52 1						+	
Gruppe 53								
S midhurst	53 L z_{39}						+	
S humber	53 z_4							+
Gruppe 55								
S tranoroa	55 k z_{39}					+		
Gruppe 56								
S artis	56 b					+		
Gruppe 57								
S locarno	57 z_{29} z_{42}					+		
S manombo	57 z_{39} e n					+		
Gruppe 58								
S basel	58 L 1					+		
Gruppe 59								
S betioky	59 k (z)					+		
Gruppe 60								
S luton	60 z e n			+				

Zeichenerklärung Pat = Patient Ges = Gesunder ? = keine Angaben W = Warmblütler K = Kaltblütler N = Nahrungsmittel A = Abwasser resp Wasser * = aus Afrika und Madagaskar

Von den 131 *Salmonella-species* des *sub-genus* II wurden 74 in Afrika und Madagaskar oder in Europa aus afrikanischem Material isoliert. Die afrikanischen Stämme kommen zum grössten Teile aus Südafrika. Es sei betont, dass keine *sub-genus* II-*species* im Congo-Gebiete isoliert wurden, obwohl wir im Laufe der Jahre ein grosses Congo-Material untersucht haben. Nur *S. bulawayo* gehört zum *sub-genus* II, wurde aber aus einem Esel in Süd-Rhodesien isoliert.

Von den 48 menschlichen Kulturen wurden 33 aus Patienten gezüchtet und zwar meist aus Enteritis-Fällen.

Von den 58 tierischen Kulturen wurden 12 aus Warmblütlern und 46 aus Kaltblütlern isoliert. Die überwiegende Zahl der tierischen Kulturen stammt also von Kaltblütlern, meist von Schildkröten, Eidechsen und Schlangen.

Von den 25 Kulturen aus Nahrungsmitteln stammen 10 aus Fleisch, und zwar meist aus importiertem Fleisch aus Bechuanaland, aus Australien (Känguruh-Fleisch) oder aus Argentinien. Die anderen Kulturen aus Nahrungsmitteln stammen aus Angola Fischmehl, aus importierten Eiern (Südafrika oder China) oder anderen Produkten.

In Bestätigung früherer Erfahrungen kommt also die Mehrzahl der *sub-genus* II-Kulturen aus Südafrika, meist aus Kaltblütlern oder importierten Nahrungsmitteln wie Fischmehl, Eiern etc.

In Dänemark wurden im Laufe der letzten 30 Jahre keine Fälle oder Ausbrüche von Nahrungsmittelvergiftung (akute Enteritis), die durch *sub-genus* II-*species* bedingt waren, festgestellt.

Auch in Frankreich wurden im Laufe der letzten Jahre keine Ausbrüche von Nahrungsmittelvergiftung durch *sub-genus* II festgestellt. Das Entsprechende gilt auch für die Bundesrepublik Deutschland und West-Berlin sowie für die Niederlande, in denen nur vereinzelte Fälle von *Salmonella sub-genus* II-Enteritis vorkamen.

Da auf Grund einer persönlichen Mitteilung von Dr. *Bokkenheuser*, Südafrika, Kulturen des *Salmonella sub-genus* II häufig bei gesunden Menschen isoliert werden und in Südafrika ubiquitär sind, so muss man bei der Beurteilung von Befunden bei südafrikanischen Patienten vorsichtig sein. Man kann daher aus obiger Tabelle nicht folgern, dass die aus Patienten isolierten *sub-genus* II-*species* in allen Fällen die Erreger der betreffenden Erkrankungen gewesen sind.

Auf Grund ausgedehnter Erfahrungen der europäischen und nordamerikanischen *Salmonella*-Laboratorien spielen die *species des sub-genus* II praktisch keine Rolle als Ursache von Nahrungsmittelvergiftungen. Trotzdem sind sie wie alle *Salmonella-species* als pathogen zu betrachten und können unter bestimmten Bedingungen Nahrungsmittelvergiftungen oder Kontakt-Infektionen auslösen. So sind z.B. kleine Kinder gefährdet, wenn sie mit Schildkröten, die derartige *Salmonella-species* des *sub-genus* II enthalten, spielen. Es sind wiederholt Fälle von Enteritis bei Kindern, die Kontakt mit Schildkröten hatten, festgestellt worden.

Abgesehen hiervon können wir aber sagen, dass *Salmonella* Infektionen mit *species* des *sub genus* II nur von ganz untergeordneter Bedeutung sind, sodass es sowohl aus klinischen als auch aus epidemiologischen Gründen genügt, die serologische Diagnose mit Hilfe des vereinfachten *Kauffmann-White*-Schemas zu stellen. Wie man aus der Tabelle sieht, kann man in der überwiegenden Zahl der Fälle die einzelnen *species* des *sub genus* II bereits mit Hilfe des vereinfachten K.W. Schemas von einander differenzieren. In speziellen Fällen, die eine nähere Analyse erfordern, kann man natürlich jeder Zeit das originale K.W. Schema anwenden.

Im Routine Betriebe des Untersuchungsamtes kann die Diagnose *Salmonella sub genus* II ohne Schwierigkeiten mit Hilfe biochemischer Methoden gestellt werden. Hat man die Verflüssigung der Gelatine sowie das typische Verhalten in organischen Säuren festgestellt, so kann an der Diagnose *sub genus* II kein Zweifel sein, sofern es sich überhaupt um *Salmonella* Kulturen handelt.

In Ergänzung der Arbeit *„Zur Serologie des Salmonella Sub-Genus II"* seien folgende Namen mitgeteilt:

S. wynberg = 1,9,12 : z_{39} : 1,7
S. parow = 3,15 : g,m,s,t : –
S. limbe = 13,22 : g,m,t : –
S. roggeveld = 51 : – : 1,7

ZUSAMMENFASSUNG

Es wird über das Vorkommen und die Bedeutung der *Salmonella sub genus* II *species* berichtet. Diese Kulturen sind bisher hauptsächlich aus Afrika (Süd- und Ost-Afrika) und aus Madagaskar sowie aus importierten Nahrungsmitteln isoliert worden. Die meisten der tierischen Kulturen wurden aus Kaltblütlern wie Schildkröten, Eidechsen und Schlangen isoliert.

Als Erreger von Nahrungsmittelvergiftungen (akute Enteritis) sind *sub genus* II Kulturen bisher in europäischen Ländern wie Frankreich, Deutschland, Holland, Skandinavien etc. entweder gar nicht oder ganz vereinzelt festgestellt worden. Es ist deshalb aus klinischen oder epidemiologischen Gründen berechtigt, derartige *species* nur mit Hilfe des vereinfachten *Kauffmann-White* Schemas zu diagnostizieren.

In einer Tabelle sind alle bis Ende 1962 bekannten *Salmonella-species* des *sub genus* II mit ihren vereinfachten Formeln, unter Angabe der Herkunft zusammengestellt.

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WEITERE SALMONELLA-SPECIES DES SUB-GENUS II (1963)

Von

F KAUFFMANN

Eingegangen 3 1 64

Im Jahre 1962 haben *F Kauffmann & R Rohde* vorgeschlagen, alle neuen *Salmonella species* der *sub genera* II und IIII mit Hilfe des vereinfachten *Kauffmann-White-Schemas* zu bestimmen *Obwohl* dieser Vorschlag auf Widerstand stiess, sind wir von seiner Berechtigung überzeugt und halten deshalb hieran fest Auch betrachten wir es als *uberflussig, neue species des sub-genus II mit Namen zu bezeichnen*

Zur Begrundung dieses Standpunktes sei auf eine vorhergehende Mitteilung von *F Kauffmann & A Petersen "Über das Vorkommen und die Bedeutung der Salmonella Sub-Genus II Species"* verwiesen Hieraus geht hervor, dass in Danemark im Laufe der letzten 30 Jahre keine menschlichen Erkrankungen durch *sub-genus*-II *species* festgestellt wurden Auch in anderen europaischen Ländern, wie Frankreich, Holland und West Deutschland, sind keine Ausbruche von Nahrungsmittelvergiftung, sondern nur ganz vereinzelte Falle von Enteritis, die durch *sub genus* II *species* bedingt waren, gefunden worden

Aus klinischen und epidemiologischen Grunden ist es daher im allgemeinen ausreichend, diese *species* nur mit Hilfe des vereinfachten K W Schemas zu diagnostizieren

In der Tabelle 1 sind die im Jahre 1963 festgestellten *species* des *Salmonella sub genus* II mit ihren vereinfachten Formeln und dem biochemischen Verhalten angegeben In der Tabelle 2 sind die vollständigeren Formeln und Namen zusammengestellt

TABELLE 1

Salmonella Species des Sub Genus II

Nr	Vereinfachte Formel	Ara	Dul	Ino	Rha	Tre	Xyl	Gly	H_2S	Gel	d	l	i	Cit	Muk	Mal
1554	C b e n z_{15}	+	+	—	+	+	+	++	+	+	×	—	—	+2	+	+
1628	C z z_{42}	+	+	—	+	+	+	++	+	+	×	—	—	+2	+	+
1544	D z_4 z_{23} z_4	+	+	—	+	+	+	++	+	+	×	—	—	+2	+	+
1636	11 G -	+	—	—	+	+	+	—	+	+	—	—	—	+-	—	—
1568	13 G	+	+	—	+	+	+	++	+	+	×	—	—	+2	+	+
1550	16 z_4 -	+	—	—	+	+	+	—	+	+	×	—	—	+-	—	—
1549	30 G	+	+	—	+	+	+	++	+	+	—	—	—	+-	+	+
1600	40 G	+	—	—	+	+	+	++	+	+	—	—	—	—	—	+
1575	43 G z_{4}		+	—	+	+	+	++	+	+	×	—	—	+2	+	+
1565	44 z_4	+	—	—	+	+	+	—	+	+	×	—	—	+-	—	—
1631	47 z l	+	+	—	+	+	+	++	+	+	×	—	—	+-	+	—
1625	48 d l	+	+	—	+	+	+	++	+	+	×	—	—	+2	+	+
1560	48 d z_4	+	—	—	+3	+	+	++	+	+	×	—	—	+2	+	+
1607	50 G l	+	+	—	+	+	+	++	+	+	×	—	—	+-	+	+
1503	50 z_4	+	—	—	+	+	+	++8	+	+	—	—	—	+2	—	—
1573	51 z_4	+	—	—	+	+	+	—	+	+	×	—	—	+2	—	—
1580	61 i z	+	—	—	—	+	+	++	+	+	×	—	—	+2	—	+

Zeichenerklärung Ara = Arabinose Dul = Dulcit Ino = Inosit Rha = Rhamnose Tre = Trehalose Xyl = Xylose Gly = *Stern*s Glycerinfuchsin Bouillon Gel = Gelatine d = d Tartrat l = l Weinsäure *i* = *i* Weinsäure Cit = Natrium Citrat Muk = Mukat, Mal = Natrium Malonat Arabinose bis Xylose + = positiv nach 1 Tage — = negativ nach 30 Tagen *Stern s Glycerinfuchsin* Bouillon ++ = Ria nach 1 2 Tagen, — = negativ nach 8 Tagen Gelatine + = langsam positiv in Ferrochlorid Gelatine und schnell positiv im *Kohn Lautrop* Test Organische Säuren + = *positiv nach 1 Tage* +2 = positiv nach 2 Tagen × = spät und unregelmässig positiv oder negativ — = negativ nach 14 Tagen

TABELLE 2
Salmonella-Species des Sub Genus II

Nr	Körper-Antigene	Geissel Antigene		Name
		1 Phase	2 Phase	
1554	6 7	b	e n x z_{42}	
1628	6 7	z	z_{42}	
1544	(9) 46	z_4 z_{24}	z_{35} z_{42}	S *maarssen*
1636	*11*	g (p) z_{51}	—	
1568	1 13 23	g (m) t	—	
1550	16	z_4,z_{32}	—	
1549	30_1	f g p t	—	
1600	40_1 40_2	g p	—	S *maartendijk*
1575	43	g s (t)	z_{42}	S *mosselbay*
1565	44	z_4,z_{32}	—	
1631	47_1 47_3	z_6	1 6	
1625	48_1 48_2	d	1	
1560	48_1 48_2	d	z_6	S *hagenbeck*
1607	50_1 50_2	g m s t	1 5	
1593	50_1 50_2 50_3	z_4,z_{23}	—	
1573	51	z_4 z_{23}	—	S *harmelen*
1589	61	i	z	S *eilbek*

Betreffs kultureller Methoden sei auf die Mitteilung von F Kauffmann *"Zur Differentialdiagnose der Salmonella Sub-Genera I, II und III"* und betreffs Serologie auf die Arbeit von F *Kauffmann "Zur Serologie des Salmonella Sub-Genus II"* verwiesen Es sei hervorgehoben dass in serologischer Hinsicht alle *3 sub-genera* in einem einzigen Antigen-Schema, dem vereinfachten *Kauffmann-White-Schema* zusammengefasst und alle als *Salmonella-species* betrachtet werden Die Unterteilung in *sub genera* betrifft also nur das vergarungsmässige Verhalten und ist von untergeordneter Bedeutung, da eine scharfe Trennung in biochemisch definierte *sub genera* nicht moglich ist Nur mit Hilfe der Serologie ist eine Einteilung in gut definierte *species* = *Gruppen verwandter, sero-fermentativer Phag Typen* möglich

In Erganzung der Tabelle 1 sei noch erwahnt, dass von den 17 neuen *species* des *sub genus* II 5 zu den bereits beschriebenen "atypischen *sub genus* II *species*" gehoren (1636, 1565, 1593, 1573 und 1550) Sie verhalten sich negativ in Dulcit, Mukat und Malonat, spalten Salicin (mit Ausnahme von 1550) und geben im KCN-Teste eine positive Reaktion

Von den bisher bekannten "atypischen *sub genus* II-*species*" enthalten 3 den G-Komplex und 7 den z_4-Komplex Wie schon fruher gesagt, wird es wahrscheinlich bald zweckmässig sein, diese "atypischen" *species* in einem besonderen *sub-genus* zu vereinen

Eine weitere *species* (1600) spaltete Sorbit nach 4 Tagen und Lactose verzogert Sie war gleichzeitig in Dulcit und Mukat negativ Nr 1589 war ebenfalls in Dulcit und Mukat negativ Nr 1544 spaltete Salicin nach 1 Tage Nr 1625 Lactose nach 4 Tagen und Nr 1560 griff Dulcit nicht an

S. eilbek = 61 : i : z wurde auch im Supplement VII zum K.-W.-Schema angegeben, da es sich hierbei um eine neue O-Gruppe handelt.

ZUSAMMENFASSUNG

Es wird über 17 neue *species* des *Salmonella sub-genus* II, die im Jahre 1963 festgestellt wurden, berichtet.

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SUPPLEMENT ZUM KAUFFMANN WHITE-SCHEMA (VII)

Von

F KAUFFMANN

Eingegangen 3 i 64

Diese Veroffentlichung ist das 3 Supplement zum *Kauffmann White* Schema in dem Buche *Die Bakteriologie der Salmonella Species* ' (*F Kauffmann* 1) und das 7 Supplement zu der Review *Das Kauffmann-White Schema* (*F Kauffmann 2*) Wahrend im 6 Supplement alle im Jahre 1962 festgestellten *Salmonella species* angegeben wurden, enthalt dieses 7 Supplement nur die neuen *species* des *sub genus 1*, mit Ausnahme von *S eilbek* da es sich hierbei um eine neue O Gruppe handelt

Nach dem Vorschlage von *F Kauffmann* & *R Rohde* „*Zur Vereinfachung der serologischen Salmonella Diagnose* werden ab 1963 nur noch *Salmonella species* des *sub genus* 1 in das originale *Kauffmann White Schema* eingefugt Diese *species* sind als *Gruppen verwandter, sero fermentativer Phag Typen definiert* und bilden die Grundlage der *modernen* Klassifikation

Im Gegensatz hierzu beginnt die *orthodoxe* Klassifikation mit den hoheren Gruppen die nur biochemisch (meist vergarungsmassig) definiert sind und keine sichere Klassifikation ermoglichen Der Satz dass die *Enterobacteriaceae* eine fortlaufende Reihe oder Serie verwandter Bakterien Typen bilden und deshalb nicht richtig klassifiziert werden konnen gilt daher nur fur die *orthodoxe* Klassifikation nicht aber fur die *moderne* *Ohne Serologie keine brauchbare Klassifikation!*

Aus dieser Tatsache geht klar hervor warum die orthodoxe Klassifikation vertreten durch *Bergey* s Manual versagte wahrend die moderne Klassifikation zum Erfolge fuhrte Es gelten auch hier die Worte der Bibel *An ihren Fruchten sollt ihr sie erkennen* Kein wirklich Sachverstandiger kann daruber im Zweifel sein dass der 1 Bericht des *Salmonella* Subcommittee s mit dem *Kauffmann White Schema* als Basis der Beginn der modernen Klassifikation war

S paratyphi B und *S typhi murium* sind zwar verwandte *species* aber serologisch durch ihre vollig verschiedenen H Antigene der 1 Phase b und i) so scharf von einander getrennt dass eine sichere Diagnose ohne Schwierigkeiten moglich ist Das Entsprechende gilt auch fur die anderen *species* des *genus Salmonella* Als einziger Uberlebender des *Salmonella* Subcommittee s schulde ich es meinen verstorbenen Freunden *H Schutze* *W M Scott* und *P Bruce White* diese

S. eilbek = 61 . i. z wurde auch im Supplement VII zum K W Schema angegeben, da es sich hierbei um eine neue O-Gruppe handelt

ZUSAMMENFASSUNG

Es wird uber 17 neue *species* des *Salmonella sub-genus* II, die im Jahre 1963 festgestellt wurden, berichtet

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und Malonat positiv waren Die Reaktion in Ammonium-Citrat war negativ

S hilversum = 30 k 1,2 war Indol-positiv und Malonat-positiv

S goulfey = 1,40 k 1,5 spaltete Sorbit nach 2 Tagen

S casablanca = 45 k 1,7 gab einen positiven β-Galaktosidase-Test

Das O Antigen von *S halle var vidin* kann mit (28_1), 28_2, 28_1 angegeben werden

LISTE DER SALMONELLA SPECIES VON 1963

Salmonella amba = 11 k L (11 k l,z_{13},z_{28})
Salmonella baguirmi = 30 y e,n,x
Salmonella breukelen = 6,8 L e,n,z_{15} (6,8 l,z_{13} e,n,z_{15})
Salmonella casablanca = 45 k 1,7
Salmonella djermaia = 28 z_{29} –
Salmonella dowgi = 50 y 1,6
Salmonella eilbek = 61 i z Atypische *species* des *sub genus* II Hier nur wegen der neuen O-Gruppe aufgeführt
Salmonella farmsen = 13,23 z 1,6
Salmonella findorff = 11 d z_6
Salmonella galil Sapiro Hirsch & Hirsch = 3,10 a e,n,z_{15} Israel Med J im Druck
Salmonella gokul = 1,51 d –
Salmonella goulfey = 1,40 k 1,5
Salmonella halle var vidin = 28+ c 1,7
Salmonella hilversum = 30 k 1,2
Salmonella honelis = 28 a e n z_{15}
Salmonella lagos = 1,4,12 i 1 5
Salmonella landala = 41 z_{10} 1,6
Salmonella lawra = 44 k e,n,z_{15}
Salmonella leiden = 13,22 z_{38}
Salmonella ljubljana = 4,12,27 k e,n,x
Salmonella malakal = 16 e,h 1,2
Salmonella menden = 6,7 z_{10} 1,2
Salmonella mons var 27 = 1,4,12,27 d l,w Wurde als Variante von *S mons* = 1,4,12 d l,w betrachtet
Salmonella nienstedten = 6 (7),(14) b l,w
Salmonella oakland = 6,7 z 1,6,7
Salmonella oevelgoenne = 28 r e,n,z_{15}
Salmonella sankt georg = 28 r(i) e,n,z_{15}
Salmonella sedgwick = 44 b e,n,z_{15}
Salmonella sladun = 4,12,27 b e,n,x
Salmonella sloterdijk = 1,4,12 27 z_{35} z_6
Salmonella synthia = 18 z_{38} –
Salmonella vaertan = 13,22 b e,n,x

Tatsachen klar hervorzuheben Deshalb habe ich auch gegen die falschen Beschlusse des Internationalen *Enterobacteriaceae*-Subcommittee's, das die *species* in orthodoxem Sinne definierte, protestiert und die richtige Einstellung des *Salmonella*-Subcommittee's betont Es handelt sich hierbei nicht um einen Streit um Worte, sondern um die zu Grunde liegende, experimentelle Methode Will man eine brauchbare Klassifikation erhalten, so muss diejenige Kategorie, durch welche die *species* sowohl biochemisch als auch serologisch definiert ist, gewahlt werden Daher ist es erforderlich, die *species* als eine Gruppe *verwandter, serofermentativer Phag-Typen zu definieren* Nach *M Planck* hangt der Fortschritt einer jeden Wissenschaft vom Einteilungsprinzip und der zu Grunde liegenden Idee ab

Das folgende *„Supplement zum Kauffmann-White Schema (VII)“* enthalt 33 neue *Salmonella-species* und 5 Varianten, die im Laufe des Jahres 1963 festgestellt wurden

Im letzten Supplement (VI) war folgendes gesagt worden „A new salmonella serotype Salmonella cook (39 z_{48} 1,5) containing an undescribed flagellar antigen“ was published by *McWhorter, Douglas & Edwards*, but not added to the K W-scheme by the author since the H antigen z_{48} could be an R-phase of *S champaign*“

Inzwischen hat *R Rohde* aus der originalen „S cook“-Kultur die 1 Phase = k isoliert und dadurch die fruhere Vermutung des Verfassers bestatigt Es handelt sich hierbei also nicht um eine neue *Salmonella species,* sondern um *S champaign* = 39 k 1,5 mit einer R-Phase = z_{48}

Der Verfasser hat die Befunde von *Rohde* bestatigt und bereits im Jahre 1961 vor der Publikation einer neuen *species* „S cook“ gewarnt Auch bei dieser Gelegenheit sei nochmals betont, dass man bei der Publikation neuer *species,* die neue Antigene enthalten, sehr vorsichtig sein soll Weder aus praktischen noch aus wissenschaftlichen Grunden ist es notwendig, neue *species* so schnell wie moglich zu publizieren Es ware ratsam, die Publikationen neuer *species* der Internationalen *Salmonella* Centrale, die jahrlich ein Supplement zum *Kauffmann-White-Schema* veroffentlicht, zu uberlassen Die Feststellung einer neuen *species* ist das Verdienst dieses Schemas und nicht des zufalligen Finders, speziell wenn er nicht einmal selbst die Antigen-Formel angegeben hat

Die neue *species S eilbek* = 61 i z wurde in die Tabelle eingefugt, da es sich hierbei um eine neue *Salmonella* O Gruppe handelt Sie gehort zum *sub-genus* II, ist aber atypisch, da sie Dulcit und Rhamnose nicht angreift Das O-Antigen 61 ist mit dem Arizona O 26-Antigen verwandlt *S eilbek* = $61_1,61_2$ und Arizona O 26 = $61_1,61_3$, doch waren die 61_3 Agglutinine sehr schwach entwickelt

S oevelgoenne = 28 r e,n,z_{15} verhalt sich biochemisch atypisch Maltose wurde spat und unregelmassig angegriffen Gelatine wurde verflussigt, d-, l-, i Tartrat und Citrat waren negativ, wahrend Mukat

, ~ 11a na ~

1

Gelat ne + = langsam pos tiv in Ferrocn ot u ue =
Kohn Lautrop Test Organische Sauren + = positiv nach 1 Tage +" = positiv nach 2 Tagen — = negativ nach 14 Tagen — = *Sub Genus* II

Salmonella vellore = 1 4 12 27 z10 z35
Salmonella wilhelmsburg var = 1 4 12 z38 – Wurde als Variante von *S wilhelmsburg* = 4 12 27 z38 – betrachtet
Salmonella wickau = 16 r(i) e n z15
Salmonella good = 21 G e n x (21 f g e n x)
Ferner wurden 2 monophasische Kulturen = 4 12 z39 – von *R Rohde und* 47 z4 z23 von *E van Oye* festgestellt und als Varianten betrachtet

Addendum zum Supplement VI

Auf Seite 345 unten ist ein Druckfehler da es *S monaline* heissen soll
Salmonella wynberg = 1 9 12 z39 1 7
Salmonella parow = 3 15 g m s t
Salmonella calabar = 1 3 10 e h 1 w
Salmonella bron = 13 22 g m e n,z15
Salmonella limbe – 13 22 g m t
Salmonella rhone – 21 c e n x
Salmonella tornow = 45 g m
Salmonella roggeveld = 51 1 7

ZUSAMMENFASSUNG

In einem Supplement zum *Kauffmann White Schema* werden 33 neue *Salmonella species* und 5 Varianten die im Laufe des Jahres 1963 festgestellt wurden mitgeteilt

LITERATUR

Kauffmann F (1 D e Bakteriolog e der Salmonella Species (Munksgaard Kopenhagen 1961
Kauffmann F (2) Das Kauffmann White Schema in Ergebnisse der Mikrobiolo 0-216 1957
Kauf Acta path
Kauf onella Dia gno t Acta path et microbiol candinav 56 341-342 1962

Species	Ara	Dul	Ino	Rha	Tre	Xyl	Gly	H_2S	Gel	d	l	i	Cit	Muk	Mal
S *sladun* = 4 12 27 b e n x	+	+	—	+	+	+	++	+	—	+	×	—	+[2]	+	—
S *mons var 27* = 1 4 12 27 d l w	+	+	+	+	+	+	++	+	—	+	+	+	+	+	—
S *lagos* = 1 4 12 i 1,5	+	+	d	+	+	+	++	+	—	d	+	+[1]	+	+	—
S *ljubljana* = 4 12 27 k e n x	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *vellore* = 1 4 12 27 z_{10} z_{35}	+	+	+	+	+	+	++	+	—	+	×	—	+	+[1]	—
Salmonella var = 4 12 z_{3}	+	+	—	+	+	+	++	+	—	+	+	—	+-	+	—
S *sloterdijl* = 1 4 12 27 z_{15} z	+	+	—	+	+	+	++	+	—	+	×	×	+-	+[4]	—
S *wilhelmsburg var* = 1 4 12 z_{38}	+	+	+	+	+	+	++	+	—	+	+	+	+	+	—
S *oakland* = 6 7 z 1 6 7	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *menden* = 6 7 z_{10} 1 2	+	+	+	+	+	+	++	+	—	+	+	+[2]	+	+	—
S *breukelen* = 6 8 l z_{13} e n z_{15}	+	+	+	+	+	+	++	+	—	+	+	+[3]	+[2]	+	—
S *niensteden* = 6 (7) (14) b l w	+	+	+	+	+	+	++	+	—	+	×	+[3]	+	+	—
S *galil* = 3 10 a e n z_{15}	+	+	—	+	+	+	++	+	—	+	+	—	+	+	—
S *fulorff* = 11 d z_{6}	+	+	+	+	+	+	++	+	—	+	+	—	+	+	—
S *amba* = 11 k l z_{13} z_{28}	+	+	—	+	+	+	++	+	—	+	×	—	+[2]	+	—
S *naertan* = 13 22 b e n x	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *leiden* = 13 22 z_{38}	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *farmsen* = 13 23 z 1 6	+	+	—	+	+	+	++	+	—	—	+[3]	+[3]	+[2]	×	—
S *malakal* = 16 e h 1 2	+	+	+	+	+	+	++	+	—	+	+	—	+	+	—
S *wichau* = 16 r(i) e n z_{15}	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *synthia* = 18 z_{38}	+	+	+	+	+	+	++	+	—	+	+	—	+	+	—
S *good* = 21 f g e n x	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *honelis* = 28 a e n z_{15}	+	+	+	+	+	+	++	+	—	+	+	—	+	+	—
S *halle var vidin* = 28+ c 1 7	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *oevelgoenne* = 28 r e n z_{15}	+	+	—	+	+	+	++	+	+	—	—	—	—	+	+
S *sankt georg* = 28 r(i) e n z_{15}	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *djermaia* = 28 z_{29}	+	+	+	+	+	+	++	+	—	+	+	+[2]	+	+	—
S *hilversum* = 30 k 1 2	+	+	+	+	+	+	++[5]	+	—	+	+	+	+	+	+
S *baguirmi* = 30 y e n x	+	+	+	+	+	+	++	+	—	+	+	+[2]	+	+	—
S *goulfey* = 1 40 k 1 5	+	+	—	+	+	+	—	+	—	+	—	—	+-	+	—
S *landala* = 41 z_{10} 1 6	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S *sedgwick* = 44 b e n z_{15}	+	+	+	+	+	+	++	+	+	+	—	—	+[2]	+	—
S *lawra* = 44 k e n z_{15}	+	+	+	+	+	+	++	+	—	+	×	—	+	+	—
S *casablanca* = 45 k 1 7	+	+	—	+	+	+	++	+	—	×	—	—	+[2 3]	—	—
Salmonella var = 47 z_{4} z_{23}	+	+	+	+	+	+	++	+	—	+	+	+[2]	+	+	—
S *dowgi* = 50 y 1 6	+	+[3]	—	+[2]	+	+	—	+	—	—	—	—	+[2]	+[2]	—
S *gokul* = 1 51 d	+	+		+	+	+	++[6]	+	—	+	—	—	+	+	—
*S *eilbek* = 61 i z	+	—	—	—	+	+	++	+	+	×	—	—	+[2]	—	+

n 1 M ammonium formate pH 6 8 and ap
ormate was run through and then distille
formate
, and 4M

Fractions positive in the serologi
dialysed and freeze dried Then th
preparation is referred to as *purified antigen A*

Both purified antigen A and extract A preparation were digested with trypsin (*Novo Copenhagen*) at pH 7 2 and 37° C

Hydrolysis was performed in glass stoppered tubes in

1 0 1 N HCl for 2 hrs at 100° C.
2 3 N HCl for 3 hrs at 100° C.
3 6 N HCl for 18 hrs at 105° C.

to dryness *in vacuo*
chromatograms with
e run in the following

systems

Spray Reagents

a for the detection

b

c and amino sugars (11

d Elson Morgan reagents modified for the detection of amino sugars on paper chromatograms (29)

e Isatine (24) a specific and sensitive reagent for proline and hydroxiproline

Paper electrophoresis was performed in veronal buffers at pH 8 6 and 8 9 (ionic strength 0 1) according to *Consden & Stanier's* method (6) The dimension of the Whatman No 1 paper was 15 0 × 30 0 cm The voltage was 500 and the time 180 mins Pieces of paper 1 cm in width were cutt off perpendicularly to the length and each piece was eluted with distilled water The extracts were tested for serological activity by the ring test and agar precipitation and read spectrophotometrically at 260 and 280 mμ

Immunoelectrophoresis was carried out with LKB 6800 A Immunoelectrophoretic Equipment according to the description in the Operating Manual

Analytical Methods

&

Hanes (12)

Hexosamines were determined by the method of *Rondle & Morgan* (31)

Specific rotation was measured in a Zeiss Circle Polarimeter (16)

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IMMUNOCHEMICAL STUDIES ON ANTIGEN PREPARATIONS FROM STAPHYLOCOCCUS AUREUS

I *Isolation and Chemical Characterization of Antigen A*

By

ARNE GROV, BERIT MYKLESTAD and PER OEDING

Received 6 i 64

An antigen present in crude extracts of *Staph aureus* strains which reacted serologically with all normal, human sera, was described by *Jensen* in 1958 (18, 19) The substance, designated antigen A, was considered to be a previously unknown polysaccharide It gave a strong zone on agar gel precipitation *Jensen* et al (20) found that extract A contained at least three components antigen A, a toxin (D), and a heterogenetic antigen of the Rantz type

Later *Lofkvist & Sjoquist* (26) demonstrated that the serologically active material in antigen A is not a polysaccharide, but a protein They also found that antigen A is serologically identical to a protein antigen (fraction B) isolated from a *Staph aureus* strain by *Verwey* in 1940 (33)

As antigen A has not been isolated in a pure state, the intention of the present study has been to purify the antigen and to characterize it chemically and serologically The serological examinations will be reported in a subsequent paper

MATERIALS AND METHODS

Strain *Staph aureus* strain Cowan I (NCTC 8530) was used throughout this investigation The strain has a high content of antigen A Its serological pattern is *a m* and the phage pattern 52/52A/80

Growth and harvesting 18 hrs cultures of the strain on nutrient agar in Petri dishes of 14 cm diameter at 37° C were harvested by scraping with a glass rod

Fractionation Methods

I Extraction and purification were performed exactly according to *Jensen*'s procedure (19) The preparation is referred to as *extract A*

II The extractions were carried out as in I but precipitation with trichloro acetic acid and ethanol were excluded The acid precipitates were collected dissolved in distilled water and dialyzed against a continuous flow of tap water for two days and finally against distilled water for two days The dialysis was carried out in cellophane tubes (Kalle & Co Wiesbaden)

The system was arranged to give a continuous increase in the ammonium formate gradient up to a molarity of one, followed by stepwise elution with M, 2M and 4M ammonium formate (pH 6 8)

The fractions were examined spectrophotometrically in a Unicam SP 500 spectrophotometer at 260 and 280 mμ, by ring test precipitation and by agar precipitation Fractions positive in the serological tests were collected concentrated *in vacuo* dialysed and freeze dried Then the column fractionation was repeated once This preparation is referred to as *purified antigen A*

Both purified antigen A and extract A preparation were digested with trypsin (Novo Copenhagen) at pH 7 2 and 37° C

Hydrolysis was performed in glass stoppered tubes in

1 0 1 N HCl for 2 hrs at 100° C
2 3 N HCl for 3 hrs at 100° C
3 6 N HCl for 18 hrs at 105° C

Paper chromatography The hydrolysates were evaporated to dryness *in vacuo* and subjected to chromatographic analysis Circular paper chromatograms with Whatman No 1 filter paper as the stationary phase (10) were run in the following systems

A *Iso* propanol 2 N HCl (65 35) (22)
B BuOH HAc H_2O (4 1 1) (23)
C EtAc Py H_2O (40 11 6) (8)
D PrOH NH_3 d 0 91 (6 4 4) (1)
E PhOH H_2O (4 1 w/v) (5)
F MeCOEt Py H_2O (70 15 15) (32)

Spray Reagents

a Aniline hydrogenphthalate in water saturated butanol (30) for the detection of aldohexoses and aldopentoses
b Na periodate benzidine for sugar alcohols (4)
c Ninhydrin 0 5 per cent in acetone for the detection of amino acids and amino sugars (11)
d Elson Morgan reagents modified for the detection of amino sugars on paper chromatograms (29)
e Isatine (24) a specific and sensitive reagent for proline and hydroxyproline

Paper electrophoresis was performed in veronal buffers at pH 8 6 and 8 9 (ionic strength 0 1) according to *Consden & Stanier's* method (6) The dimension of the Whatman No 1 paper was 15 0 × 30 0 cm The voltage was 500 and the time 180 mins Pieces of paper 1 cm in width were cutt off perpendicularly to the length and each piece was eluted with distilled water The extracts were tested for serological activity by the ring test and agar [illegible] and read spectrophotometric

Immunoelectrophoretic [illegible]ual

Analytical Methods

21)
& [illegible] *Fiske* [illegible] *Young* (35)

Reducing sugars were determined by Hagedorn Jensen's method as modified by *Hanes* (12)

Hexosamines were determined by the method of *Rondle & Morgan* (31)

Specific rotation was measured in a Zeiss Circle Polarimeter (16)

Deoxypentoses and pentoses were examined by *Dische*'s diphenylamine test (7) and *Bial*'s test (3), respectively.

The qualitative *Molisch test* for carbohydrates and the qualitative *biuret test* for proteins were carried out as described in (14). Determination of *sialic acids* was performed according to *Warren*'s thiobarbituric acid method (34).

Quantitative determinations of the amino acids were performed as described in (11) using a Unicam SP 500 spectrophotometer.

Serological Methods

Immune serum against strain Cowan I was produced by intravenous injections of formalin killed bacteria as described by *Oeding* (28). A pool of 10 normal human sera was used in all experiments.

The *ring test precipitation* was carried out according to the method used by *Haukenes et al.* (14), and the *agar precipitation* technique as the one used by *Haukenes & Oeding* (15).

The *haemagglutination test* was performed essentially as described by *Morse* (27). A 1:40 000 solution of tannic acid was used for tanning the sheep cells.

RESULTS

Serologically active material was liberated just after the gradient was started and was out of the column before a M concentration of ammonium formate was reached. Some ultraviolet light absorbing material goes straight through the column. Absorption at 260 and 280 mμ also occurs in the region of active material, but the majority of the light absorbing material was liberated after a concentration of 0.5 M ammonium formate had been reached.

At the second column fractionation, the active material was liberated in the same region as before, and was now almost free from ultraviolet light absorption. A marked increase in ring test titre was observed. Obviously, very little ultraviolet light absorbing material is associated with the serologically active material. The absorption spectrum is shown in Fig. 1.

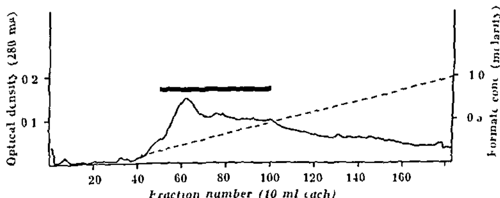


Fig. 1.

Rechromatography of antigen A on DEAE cellulose at pH 6.8.

—— 280 mμ

- - - Formate concentration

▬ Antigen A present in the eluate on ring test.



Fig 2

Schematic drawing of the lines given by the two antigen preparations against Cowan I antiserum and human serum on agar gel diffusion

CI Cowan I antiserum undiluted
H Human serum undiluted
I Extract A 1 mg/ml
II Purified antigen A 1 mg/ml

The purified antigen A preparation obtained was a white and very light material It was slightly soluble in distilled water (pH 6), but completely soluble at about pH 7 5

The purified antigen A gave a ring test titre of 1 80 with Cowan I antiserum using a primary concentration of 1 mg ml, whereas the titre of extract A was only 1 10 On agar precipitation both products gave a double line with Cowan I antiserum and a single line with human serum As shown in Fig 2, the precipitation lines of purified antigen A give a reaction of identity with those of extract A

Samples of 5 mg each of purified antigen A, extract A, and a nucleic acid preparation (from Thymus Gland, BDH) were hydrolysed in 0 1 N hydrochloric acid and examined for purines and pyrimidines by circular paper chromatography in system A using adenine and guanine as controls The spots were located on the chromatograms by means of an ultraviolet lamp Both extract A and the nucleic acid preparation showed adenine and guanine, whereas nothing could be observed in the purified antigen A sample

3 N hydrochloric acid hydrolysates were examined chromatographically for aldohexoses and aldopentoses in system B and C Extract A showed bands corresponding to authentic specimens of ribose and glucuronic acid In the 0 1 N hydrochloric acid hydrolysate of the same compound were observed two pentose coloured bands One of these was a weak ribose band while the more central one was shown to be an unhydrolysed complex consisting of ribose and uronic acid The purified antigen A preparation had two very weak bands showing movements identical to those of authentic samples of xylose and glucose

Examinations for sugar alcohols were performed chromatographically with solvent system D and spray reagent b Glycerol and hydrolysed samples of a ribitol teichoic acid and a glycerol teichoic acid, were used as references (25) In extract A ribitol and an unidentified band were detected, whereas nothing could be seen in the purified antigen A

Both 3 N and 6 N hydrochloric acid hydrolysates were tested for hexosamines using solvent system B and spray reagent d Identical

weak bands appeared in both antigen preparations showing a slower movement than those of glucosamine and galactosamine

The sialic acid determinations gave a value of 0 05 per cent using equation 2 in Warren s assay

Ten amino acids were detected in 6 N hydrochloric acid hydrolysates of both antigen preparations The amino acids listed in Table 1 showed *identical movements* as those of authentic samples in systems B E and I

TABLE 1

Purified Antigen A Analytical Data

	Results in orig nal conc	Per cen
Biuret	+	
Bial	—	
Dische	—	
Molisch	+	
Specific rotation $[\alpha]_D$ 21° C	—50 66	
Nitrogen		15 38
Phosphorus		0 00
Reducing sugars (as glucose)		5 00
Hexosamines (as glucosamine)		0 00
Sialic acid		0 05
Lys		15 6
Asp A		14 8
Ser		5 9
Gly		12 3
Glu A		14 7
Thr		trace
α Ala		3 5
Pro		12 3
Val		6 5
Leu		7 4

In accordance with the findings by *Lofkvist & Sjoquist* (26) our purified antigen A preparation migrated slowly towards the anode on electrophoresis in the buffers used No fractionation of the material occurred and the *serological reactions* were unchanged The material seemed to be homogenous on immunoelectrophoresis also The double line observed on agar precipitation with serum Cowan I appeared as one line on immunoelectrophoresis

Using the tanned cell haemagglutination technique however the purified antigen A preparation was shown to contain a sensitizing substance which is not identical to the precipitinogen Extract A in addition to these two antigens had a substance which sensitizes sheep cells directly Serological details will be given in a subsequent report

Tryptic digestion led to loss of precipitability in both antigen preparations and loss of the ability to sensitize tanned sheep cells whereas the factor sensitizing normal sheep cells remained intact

By fractionating 250 mg of extract A on the column as described

above, we succeeded in separating the two different sensitizing substances

I Fractions 1–52 No precipitinogen Sensitized only tanned sheep cells

II Fractions 53–75 Precipitinogen present Sensitized both normal and tanned sheep cells

III Fractions 158–170 No precipitinogen Sensitized only normal sheep cells

All the three groups were, after concentration *in vacuo*, first hydrolysed in 2 N hydrochloric acid for 3 hrs at 100° C and then in 6 N hydrochloric acid for 18 hrs at 105° C

Chromatographical examination of the sugars in the 2 N hydrolysates showed ribose and glucuronic acid in group III, and weak hexose and pentose bands in group II, whereas nothing could be observed in group I

Examination for amino acids in the 6 N hydrolysates gave the following results

Group I Lys, Asp A (trace), Ser, Gly, Glu A, and Ala (weak)

Group II The same ten amino acids as found in the two antigen A preparations

Group III Only small amounts of Lys, Gly, and Ala were detected

Quantitative determinations of the amino acids showed that these constitute about 99 per cent of the purified antigen A Threonine was present in amounts too small for quantitative determination

All the data obtained from the quantitative as well as the qualitative analyses of purified antigen A are listed in Table 1

DISCUSSION

Increasing serologic activity with decreasing ultraviolet light absorption indicates that very little of the absorption is associated with serologically active material The negative findings for purines and pyrimidines together with negative Bial and Dische tests exclude the presence of nucleic acids in purified antigen A This is also in accordance with the absence of phosphorus

Considering the extremely high sensitivity of the aniline hydrogenphthalate reagent and the minute amount of free sugars found on the chromatograms, it is likely that these sugars are due to traces of contaminants In addition the trace amount of hexosamine detected on chromatograms was not found when *Rondle & Morgan's* method was used It has been shown (2) that members of the sialic acid family of compounds occur in bacteria incorporated in conjugated protein, and it is likely that the observed traces of hexosamine and free sugars in purified antigen A are due to degradation products of the sialic acid

demonstrated The relatively high reducing power observed must be related to the protein and amino acids.

The great variety of amino acids and the high nitrogen percentage *indicate that the major part of the antigenic material is composed of protein.* This is confirmed by the quantitative determinations, that show that the amino acids present account for 99 *per cent of the purified* antigen A. Likewise, the nitrogen bound to these amino acids, is in good agreement with the nitrogen found by the micro-Kjeldahl *method* (The accuracy of the quantitative method used is within 5–10 *per cent*) Disappearance of both the precipitin and haemagglutinin reaction after tryptic digestion also shows that the antigenic activity is associated with the protein content *Lofkvist & Sjoquist* (26) came to the same conclusion, as they got rid of the nucleic acid part of their crude extract by electrophoresis

Calculations show that the molar ratios of the amino acids Leu.Val Pro.Ala:Gly:Ser.GluA:AspA:Lys are approximately as 1.1:2.2.3 1:2·2:2

Both column fractionation and electrophoresis seem to show that the purified antigen A preparation contains one single precipitinogen The significance of the double line regularly observed in the Cowan I immune serum will be discussed in a subsequent paper It was, however, demonstrated that an antigen is also present, which sensitizes tanned sheep cells The sensitizing activity could be separated from the precipitating one on the cellulose column Both are protein antigens, but they contain different amino acids The sensitizing and precipitating activities thus seem to be linked to separate molecules

Apparently, the amino acids Leu, Val, Pro, and Asp A contribute only to the precipitinogen Weak hydrolysis showed the last one to be terminal The antigen sensitizing tanned sheep cells seems to be composed of Lys, Gly, Ser, Glu A, and Ala, the same amino acids as are found in the hydrolysate of polysaccharide A (17) The only difference observable on the chromatograms is that the amount of Ser and Glu A in the sensitizing substance isolated from purified antigen A is relatively greater than that in polysaccharide A The mucopeptide moiety of polysaccharide A seems to be responsible for the sensitizing activity Not only in its amino acid composition, but also serologically this sensitizing substance seems to be identical to that of purified antigen A

The small amounts of amino acids found in group III on column fractionation of extract A are probably due to contaminants The antigen, sensitizing normal sheep cells, found in this group was resistant to tryptic digestion and may probably be a saccharide complex The only components observed were glucuronic acid (or galacturonic acid), ribose, and most probably the sugar alcohol ribitol

There is a striking similarity between the analytical data for our purified antigen A and those of *Verwey s* fraction B (33) Like *Lofkvist* & Sjoquist (26) we are of the opinion that *Verwey s* protein antigen

is identical with antigen A, later described by *Jensen* For this reason, and to avoid confusion with polysaccharide A and antigen a, the precipitinogen in antigen A should be designated *protein A*

SUMMARY

Antigen A has been extracted from *Staph aureus* strain Cowan I and purified on an ion exchange column Chemical analyses showed that the purified antigen material contained 15 38 per cent of nitrogen, whereas no phosphorus could be detected Circular paper chromatography of hydrolysates revealed 10 amino acids, Leu, Val, Pro, Ala, Thr, Gly, Ser, Glu A, Asp A, and Lys Quantitatively the amino acids constituted 99 per cent of the antigen material As the precipitinogen described as antigen A is a protein probably identical to *Verwey's* protein fraction, it is proposed that it should be called *protein A*

The purified material also contained an antigen sensitizing tanned sheep cells while the crude extract contained an additional antigen sensitizing normal sheep cells The two sensitizing substances were separated on an ion exchange column The former is a protein while the latter is a polysaccharide

The serological investigations are to be reported in a subsequent paper

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THE PASSAGE OF PARENTERAL PENICILLIN INTO THE CEREBROSPINAL FLUID IN BACTERIAL MENINGITIS OF VARYING GENESIS

An Experimental Investigation on Rabbits

By

Arne Lithander and Brita Lithander

Received 5 xi 63

Clinical experience has revealed that the blood cerebrospinal fluid barrier is scarcely permeable by penicillin under normal circumstances With infection of the meninges on the other hand, the barrier is damaged and the passage of for example, penicillin from blood to cerebrospinal fluid becomes much freer Some workers have pointed out that the permeability is greater when meningitis is acute than when it is regressing Whether there is any difference in permeability with different kinds of infection in the meninges has not been established by clinical st[illegible] up to the present However *H V Smith* (1) noted that tubercul[illegible]
regard t[illegible]
barrier [illegible]
into the cerebrospinal fluid is negligible in syphilis in comparison with acute forms of meningitis

The literature contains practically no reports of experimental investigations on the effect of various acute infections of the meninges on the permeability of the blood cerebrospinal barrier by penicillin *W Finck* (2) compared the permeability of the blood cerebrospinal fluid in eight rabbits with meningitis produced experimentally with staphylococci and with streptococci but these experiments referred only to the passage of P[32] No difference between staphylococcic and streptococcic meningitis was found in these experiments

Lithander & Lithander (3 4) studied the influence of staphylococcic meningitis on the permeability of the blood cerebrospinal fluid by penicillin in rabbits They found that the penicillin concentration in the cerebrospinal fluid depended on the intensity of the clinical picture, the concentration of penicillin in the plasma and the interval which elapsed between the intravenous introduction of penicillin and the sampling of the cerebrospinal fluid The age of the experimental animals on the other hand appeared to be of no significance

The purpose of the present investigation was to study the passage of penicillin from blood to cerebrospinal fluid in meningitis of varying bacterial genesis and the differences which may characterize different infections. Meningitis was produced with α-, β- and γ-streptococci, pneumococci, and meningococci. The results were compared with the results in staphylococcic meningitis secured from earlier investigations (3). The experiments with staphylococci were expanded in the present investigation in order to obtain all the groups of symptoms which developed in the experiments with the other bacterial species.

METHODS AND MATERIALS

The rabbits used weighted between two and three kilograms. They were given meningitis by intracisternal injections of bacterial suspensions as previously described (3). For the injections we used suspensions of α-, β- and γ-streptococci, pneumococci and meningococci, which had been washed repeatedly in physiological saline solution. To produce meningitis of varying intensity the number of bacteria in the doses was varied, but the volume was always 0.5 ml. The doses usually comprised 0.25–4 billion β-streptococci group A (4 strains), 0.2–2 billion α-streptococci (5 strains), 0.5–6 billion γ-streptococci (5 strains), 0.05–0.5 billion pneumococci typ III and 22 A (2 strains of each), and 2–10 billion meningococci (4 strains) respectively. The bacteria were injected into the cistern immediately after the removal of 0.5 ml of cerebrospinal fluid.—The day after the injection the rabbit was given 20 000 units of intravenous benzyl penicillin per kg of body weight.[1] Samples of cerebrospinal fluid, blood and brain were removed 30 minutes after administration of penicillin. The penicillin concentration in the samples and the concentration of leucocytes and erythrocytes in the cerebrospinal fluid were then determined. Samples of brain tissue were retained for histologic examination. The technique for the foregoing procedures was the same as described in an earlier report (3).

In the experiments with pneumococci, streptococci and meningococci the classification according to symptom groups was done on the same grounds as in the experiments with staphylococci (3). Thus group +++ comprised pronounced signs of meningitis with spasticity, nuchal rigidity and opistotonus, group ++ moderate, and group + mild symptoms of such types. In the experiments with pneumococci, streptococci and meningococci we defined still another group comprising uncertain signs of meningitis, for example the rabbit's movements were somewhat hesitant and jerky, suggesting beginning spasticity, and the possibility of nuchal rigidity could not be dismissed. This group was labelled (+). Finally the rabbits completely free from signs of meningitis were referred to group—. To have groups (+) and— represented among the rabbits infected with staphylococci also a number of rabbits were given intracisternal injections of staphylococci.

RESULTS

The intensity of the general symptoms in the various groups of symptoms corresponded to the degree of the signs of meningitis. In group —, the cerebrospinal fluid was completely or almost clear, while in the others there was cloudiness which increased with the signs of meningitis.

Table 1 and Figs. 1, 2 and 3 show the concentration of penicillin in the different organic substances, as well as the concentration of leucocytes (only in Table 1) in cerebrospinal fluid.

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[1] Benzyl penicillin is abbreviated to "penicillin" in the following. The penicillin in the present investigation was kindly supplied by AB KABI, Stockholm, Sweden.

TABLE 1

Penicillin Concentrations in Plasma, Cerebrospinal Fluid and Brain are expressed in log (100 × mean of units of penicillin per 1 ml) and the Concentration of Leucocytes in Cerebrospinal Fluid log (mean of the number per mm³) in Different Symptom Groups. In the Table these Expressions are Abbreviated to log

Variables	Bacteria	N	log	N	log	N	log	N	log	N	log	σ	t
		Groups of Symptoms											
				( )				++		+++			
Plasma	St	5	2.478	4	2.378	7	2.336	8	2.315	20	2.311	0.234	0.21
	α	7	2.308	6	2.560	8	2.448			5	2.724	0.303	1.31
	β	7	2.550	8	2.641	7	2.501	9	2.354	7	2.740	0.331	1.40
	γ	5	2.372	6	2.472	5	2.364	6	2.502	3	2.877	0.343	1.28
	M	6	2.190	11	2.405	7	2.464	4	2.333	4	2.648	0.341	1.16
	Pn	4	2.055	5	2.258	5	2.148	9	2.176	7	2.377	0.228	1.59
Cerebrospinal fluid	St	5	0.960	4	1.262	7	1.263	8	1.281	20	1.575	0.230	8.84*
	α	7	1.330	6	1.385	8	1.533	—	—	5	1.810	0.243	4.37*
	β	7	1.581	8	1.720	7	1.871	9	1.833	7	2.363	0.271	8.46*
	γ	6	1.148	6	1.431	5	1.548	6	1.466	3	[illegible]	0.182	10.28*
	M	6	1.080	11	1.346	7	1.484	4	1.582	4	1.940	0.276	6.45*
	Pn	4	0.835	5	1.082	5	1.286	9	1.551	7	1.911	0.124	60.94*
Brain	St	5	0.926	4	1.165	7	1.011	8	1.025	20	1.124	0.218	1.26
	α	7	1.113	6	1.150	8	1.016		—	5	1.100	0.204	0.03
	β	7	1.171	8	1.161	7	1.300	9	0.926	7	1.387	0.272	3.32*
	γ	5	1.020	6	1.004	5	0.958	6	1.082	7	1.400	0.228	2.05
	M	6	0.842	11	0.968	7	1.003	4	0.882	4	1.430	0.275	3.16*
	Pn	4	1.048	5	1.012	5	0.924	9	1.074	7	1.217	0.138	3.67*
Leucocytes	St	6	2.764	4	3.238	7	3.133	8	3.361	20	3.379	0.330	3.93*
	α	7	3.431	6	3.673	8	3.673	—	—	4	4.170	0.301	5.97*
	β	7	3.110	8	3.203	7	3.571	9	4.003	7	4.460	0.434	12.42*
	γ	5	3.366	6	3.583	5	3.752	6	3.912	3	3.963	0.446	1.42
	M	6	3.648	11	3.823	7	3.957	4	3.590	3	3.970	0.307	1.54
	Pn	4	2.528	4	2.678	5	3.282	8	3.330	6	3.537	0.471	4.49*

Variance ratio in an analysis of variance is given under t

* denotes significant trend for the penicillin concentration to increase with the intensity of the clinical symptoms of meningitis $P < 0.05$

St denotes staphylococci; α, β, γ denotes the corresponding species of *streptococci*

M denotes meningococci

Pn denotes pneumococci

The concentration of leucocytes in the cerebrospinal fluid corresponded in general to the intensity of the symptom picture (Table 1) It was thus lowest in group — and highest in group + + + The increase of the concentration of leucocytes with the meningitis symptoms was significant for α- and β-streptococci, for staphylococci and pneumococci, but not for γ-streptococci and meningococci The concentration of leucocytes in cerebrospinal fluid in the experiments with staphylococci and pneumococci was lower than with the other species of bacteria

The concentration of erythrocytes in cerebrospinal fluid was very low and had no relation to either the degree of meningitis or the concentration of penicillin in cerebrospinal fluid with any of the bacteria

There was no significant difference between the various degrees of meningitis with regard to concentration of penicillin in plasma (Table 1, Fig 1) This was true, regardless of the kind of bacteria which produced the meningitis

The penicillin concentration in cerebrospinal fluid (Table 1, Fig 2) increased significantly with the degree of meningitis for all bacterial strains The greatest increase was noted between symptom groups + + and + + + The penicillin concentration also varied considerably according to the cause of the infection In meningitis produced with β-streptococci, the penicillin concentration was at a higher level than with other bacterial causes of the infection Thus, even before clinical signs of meningitis had time to appear, *i e* in symptom group —, the

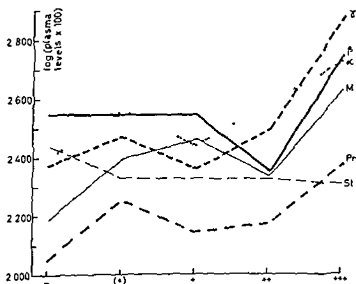


Fig 1

The concentration of penicillin in plasma in rabbits with bacterial meningitis of varying genesis 30 minutes after intravenous administration of penicillin The penicillin concentration is expressed in log (100 × mean of units per ml)

St denotes staphylococci

α, β, and γ denote the respective species of streptococci

M denotes meningococci

Pn denotes pneumococci

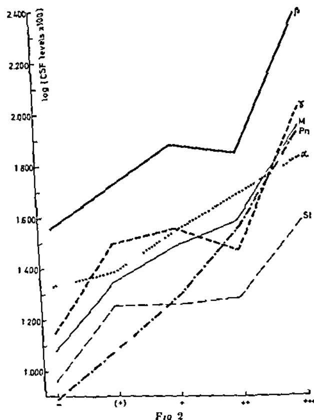


Fig 2

The concentration of penicillin in cerebrospinal fluid (CSF) in rabbits with bacterial meningitis of varying genesis 30 minutes after intravenous administration of penicillin The penicillin concentration is expressed in log (100 × mean of units per ml)

St denotes staphylococci
α β and γ denote the respective species of streptococci
M denotes meningococci
Pn denotes pneumococci

penicillin concentration was as high as in symptom group + + + with staphylococci meningitis.

In meningitis produced by meningococci, α- and γ-streptococci the penicillin concentration in cerebrospinal fluid was higher in almost all symptom groups than in infections produced by staphylococci

It has been shown previously (3), that when meningitis in rabbits was produced with staphylococci, the ratio

$$\frac{\text{penicillin concentration in cerebrospinal fluid}}{\text{penicillin concentration in plasma}} \left(\frac{F}{P}\right)$$

increased with the intensity of sympotms In the present experiments (Table 2), the same tendency was observed for β streptococci, meningococci and pneumococci

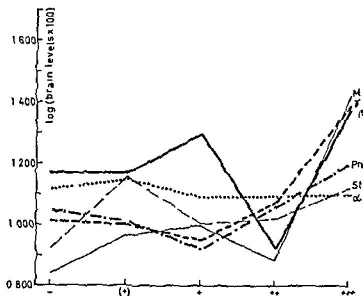


Fig 1

The *concentration of* penicillin in brain substance in rabbits with bacterial meningitis of varying genesis 30 minutes *after* intravenous administration of penicillin. The penicillin concentration is expressed *in log (100 × mean* of units per ml)
α-, β-, and γ-denote the respective species of streptococci
St denotes staphylococci
M denotes meningococci
Pn denotes pneumococci

TABLE 2

The Correlations $\left(\frac{F}{P}\right)$ *and* $\left(\frac{B}{P}\right) = \left(\frac{\textit{penicillin concentration in brain}}{\textit{penicillin concentration in plasma}}\right)$ *in per cent*

Groups of Symptoms

Bacterial species	−		(+)		+		++		+++	
	I P	B P	I P	B P	I P	B P	I P	B P	I P	B P
St	3 3	3 1	9 0	6 1	8 5	4 7	9 2	5 1	18 4	6 5
α	8 6	5 3	6 7	3 9	10 2	4 4			10 2	2 4
β	10 1	4 2	14 8	4 1	23 4	6 3	30 1	3 7	43 5	4 4
γ	6 0	4 4	10 0	4 3	15 3	3 9	9 2	3 8	10 2	3 3
M	7 8	4 5	8 7	3 7	9 6	3 5	14 1	3 5	20 0	6 2
Pn	6 9	9 8	6 7	5 7	13 7	6 0	23 7	7 9	34 2	6 9

St denotes staphylococci α β- γ-denote the respective species of streptococci
M denotes meningococci
Pn denotes pneumococci

In the symptom groups in which the signs of meningitis were most pronounced i e groups + + and + + +, there was a distinct difference between meningitis caused by β streptococci and by pneumococci, on the one hand, and the other types of meningitis on the other hand With the two first-mentioned infections, the $\frac{F}{P}$ in these symptom groups was higher than with the other infections This was particularly true

of meningitis produced by β-streptococci It is also noteworthy that, with this infection, $\frac{F}{P}$ was high even before the clinical signs of meningitis had time to develop When the infection was caused by staphylococci, $\frac{F}{P}$ in this symptom group, —, was approximately at the same level as demonstrated in uninfected control rabbits with no signs of meningitis (3)

The penicillin concentration in brain substance (Table 1, Fig 3) was low in all symptom groups When meningitis was produced by β-streptococci meningococci or pneumococci, there was a significant trend for the penicillin concentration in the brain to increase with the degree of meningitis This was not the case in the experiments with α- and γ-streptococci or staphylococci

Microscopic examination of specimens of the meninges removed from the rabbits confirmed the clinical findings Thus the intensity of the anatomic changes in the cerebral meninges in the individual rabbits was usually proportional to the clinical degree of the meningitis In the meninges of some of the rabbits without clinical symptoms of meningitis (group —) the microscopic examination revealed slight inflammation In the meninges of the other rabbits in this group there were no anatomical changes As regards the anatomical changes in the meninges there were no differences between the various bacterial species used in this investigation

DISCUSSION

In the present experimental investigations, we found differences between various bacterial species which cause meningitis with regard to the influence on the passage of penicillin from the blood into the cerebrospinal fluid The effect of β-streptococci was clearly greater than of the other bacteria used in the experiments This was true even in consideration of the concurrent penicillin concentrations in the plasma On the other hand, staphylococci appeared to have the least effect on the blood cerebrospinal barrier The cause of the unique situation of β streptococci did not appear from the investigation

Common to all the bacterial species studied was a significant increase of the penicillin concentration in the cerebrospinal fluid with the signs of meningitis Analysis revealed (Table 2) that this trend was real for β streptococci meningococci and pneumococci through all the degrees of meningitis signs, *i e from* — to + + +, as was demonstrated earlier (3) for staphylococci in the meningitis sign groups +, + +, and + + + The ability of the β streptococci to affect the blood cerebrospinal fluid barrier appeared even before the clinical signs of meningitis had become evident

The penicillin concentration in the brain showed a significant trend to increase with the meningitis signs when the infection was produced

with β-streptococci, meningococci and pneumococci This may have been a direct consequence of a correlation between the penicillin concentration in plasma and in brain substance Analysis of the ratio $\frac{\text{penicillin concentration in brain}}{\text{penicillin concentration in plasma}} \left(\frac{P}{B}\right)$ (Table 2) shows this trend to be completely dependent on the concurrent plasma concentration Hence, the penicillin concentration in the brain appears to have been independent of the intensity of the signs of meningitis

SUMMARY

1 Meningitis was produced in rabbits with different bacterial species and the passage of penicillin into the cerebrospinal fluid and brain following intravenous injection was studied

2 The various bacterial species had different effects on the blood-cerebrospinal fluid barrier The passage of penicillin into cerebrospinal fluid was most pronounced in the experiments with β-streptococci, which suggests that this bacterial species had the greatest capacity to damage the blood-cerebrospinal barrier In this infection, the blood-cerebrospinal fluid barrier appears to have been affected earlier than in the other infections studied

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GROWTH OF 17D YELLOW FEVER VIRUS AND FACTORS INFLUENCING ITS TRANSMISSION WITHIN CELL CULTURES *IN VITRO*

By

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Received 24 i 64

The relationship of cytopathogenic effects (CPE) to the spread of virus infection within a cell culture has been studied in the light of accumulating evidence (*Isaacs & Baron* 1960, *Wagner* 1963, reviewed by *Wagner* 1963) which suggests that successive cycles of virus growth may differ one from the other because of the production of interferon by infected cells Interferon limits the growth of viruses, which reduces their destructive action on the total cell population and presumably allows cells and virus to exist together in some form of equilibrium mixture as a carrier culture

This interaction between cells and viruses was studied using the percentage of infected cells as the main parameter for measuring the spread of yellow fever virus, strain 17D, in a population of susceptible cells Cell cultures infected with this virus are seldom completely destroyed and carrier cultures may be easily established (*Doherty* 1958, *Hallauer* 1959), suggesting the presence of an adaptation mechanism in infected cultures The change in the percentage of infected cells with time was found to be a sensitive indicator of interfering substances as well as virus infectivity

METHODS

Virus The 17D strain of Yellow Fever was obtained as a lyophilized yolk sac vaccine from Paris France The infectious material was inoculated into monolayers of Chang's *human liver (CHL) cells and incubated for 4 days Successive* virus seeds a
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alcohol bath and a 37° C water bath respectively to liberate intracellular virus and bring the entire cell sheet into suspension The suspension was stored at —60° C

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where the infectivity was stable for at least 3 months If the cellular material was removed by centrifugation 99 per cent of the infectivity of seeds was lost over a 2 month period at —60° C Freezing and thawing 4 times did not measurably reduce the infectivity, however, most infectious material was frozen and thawed no more than twice before use in an experiment or titration Most experiments reported here were performed with the 7th passage of 17D in CHL cells As each seed was used up more was made by using the 6th passage virus which had been stored at —60° C for this purpose

Cell cultures—Chang's human liver cells were grown in 10 ml volumes of the lactalbumin hydrolysate medium with 5 per cent horse serum in 3 oz prescription bottles and were infected when the cell concentration reached about 10^6 cells per bottle

Titration—The Virus was titrated by intracerebral (I C) inoculation of 12 to 14 gram Swiss albino mice with a volume of 0 03 ml Five mice were used per dilution and survivors were discarded after 14 days observation The dilutions were made in 10 fold increments with the lactalbumin hydrolysate medium as diluent and titrated immediately The LD_{50} was calculated by the method of Reed & Muench but the titre was estimated only to the nearest half log because of the large variations occurring with this form of titration

Infection of cultures—The cell monolayer was washed twice with 0 01*M* phosphate buffered saline (PBS) pH 7 4 and then shaken with 1 ml of the virus suspension for 1 hour at 37° C on a platform shaker (about 35 cycles per minute) After this time the cell layer was washed twice with PBS and 10 ml of fresh medium added Unless otherwise noted the medium was not changed after infection

Estimation of per cent infected cells—The following method of estimating the per cent infected cells was developed because a plaque technique with Yellow Fever virus had not been successfully worked out at the time

The infected cell layer was washed twice with PBS to eliminate extracellular virus and suspended in 5 ml 0 02 per cent versene in PBS The cells were centrifuged at 1 000 × g for 10 minutes resuspended in 1 or 2 ml lactalbumin medium depending upon the cell concentration and counted in a haemocytometer They were diluted in 10 fold increments and titrated directly in mice without being frozen and thawed 0 2 ml aliquots from each dilution tube were added to each of 5 Wasserman tubes containing about 10^5 cells growing as a stationary culture in 2 ml medium These tubes were incubated in a stationary inclined position of about 15° to the horizon for 5 days at 37° C and then examined for CPE The presence or absence of virus in each set of tubes bracketing the CPE endpoint was tested by I C mouse inoculation Since the CPE endpoint was frequently equivocal determining the presence of infectious virus increased the sensitivity of the titration because frequently virus was present in tubes which showed no CPE The Most Probable Number of infected cells in the population was calculated by the method of *Chang et al* (1958) and the percentage of infected cells estimated from the known total cell count

RESULTS

Adsorption of 17D by CHL Cells—The optimum time for adsorption of 17D by CHL cells was measured by exposing the cell layer to virus for varying periods of time and then estimating the percentage of infected cells after 24 hours incubation (Fig 1) This value actually represented the percentage of infected cells after more than one cycle of virus growth The values obtained immediately after infection (zero time) were usually very low seldom exceeding 1 per cent infected cells There was always a marked increase in the percentage of infected cells during the second cycle and this increase was found to be in proportion to the inoculum used Therefore, the 24 hour value was used in many experiments to measure the effect of varying conditions on virus infection

In Fig 1 CHL monolayers containing about 3 × 10 cells per bottle

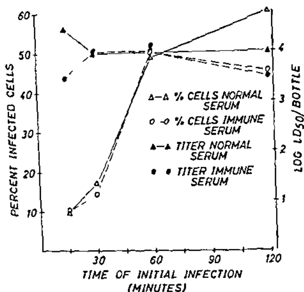


Fig 1

Effect of adsorption time on the resulting percentage of infected cells 24 hours after infection with 10^3 LD_{50} 17D virus After the adsorption times indicated the infected cell layer was washed twice with PBS and then treated with 1 ml of a 10^{-1} dilution of anti 17D human or non immune serum for 1 hour The cell layer was again washed twice with PBS and then 10 ml medium added The corresponding infectivity curves have filled in symbols

were exposed to 10^3 LD_{50} 17D in a volume of 1 ml for 15, 30, 60 and 120 minutes After these various time periods the cell layers were washed twice with PBS and 1 ml of 10 per cent anti-17D human serum in PBS was added to the cell layer for 1 hour at 37° C to neutralize adsorbed virus which might be still susceptible to antiserum The immune human serum at this dilution was capable of neutralizing at least 10^3 LD_{50} 17D in 1 hour at 37° C Another set of infected cultures were treated with non-immune human serum The serum was washed off with PBS and fresh medium added After 24 hours incubation the infected cell population was determined

The total number of cells remaining in the monolayer after this 24-hour-period was still about 3×10^6 cells per bottle and no CPE was observed The increase in the percentage of infected cells during the first 60 minutes of adsorption was identical when either immune or non-immune human serum was used After 60 minutes the cultures treated with immune serum showed no increase in the percentage of infected cells, while the cultures treated with non-immune serum showed a slight increase by 120 minutes adsorption time

A 60-minute-adsorption period was used routinely in the following experiments, since beyond this time period the rate of adsorption appeared to be slower

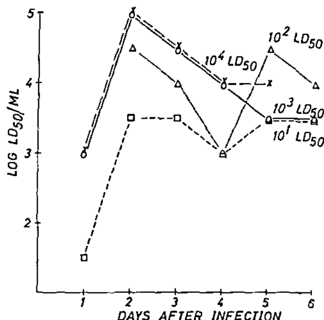


Fig 2

Resulting growth curves of 17D virus in CHL cells infected with varying concentrations of virus The titres represent the combined infectivity of cells and medium

Growth curves—Fig 2 shows the growth response of 17D in CHL cells *with inocula ranging from 10^4 to 10^1 LD_{50} The titres represent* the combined cell-associated and extra cellular infectivity Maximum titre was generally reached by the second day after infection and was followed either by a general drop in infectivity or a leveling off of the infectivity at about $10^{3.5}$ LD_{50}/ml With inocula of 10^1 LD_{50}, the titre remained fairly constant after the 2nd day instead of continuing to increase as more cells became infected Inocula of 10^3 LD_{50} and higher gave identical virus yields as though the cell cultures were saturated with virus above this inocula However, it will be apparent from the data to be presented that the percentage of infected cells varied considerably with inocula above this range It was observed, also, that when mice were inoculated I C with virus concentrations of 10^3 to 10^6 LD_{50}, they did not die before the 7th day following inoculation and the average day of death of mice inoculated with these high concentrations of virus was the same Below 10^3 LD_{50} the average day of death of the mice increased proportionally with dilution of infectivity

Fig 3 shows the variation in the percentage of infected cells with time and inoculum Although the general shape of these curves were all similar, the highest percentage of infected cells varied in time inversely with the virus inoculum The percentage of infected cells always rose and decreased sharply each within an interval of a day, the precipitous drop in the percentage of infected cells corresponding to the appearance of CPE in the culture The intra-cellular virus titre increased and decreased in a corresponding manner with the percentage of infected cells

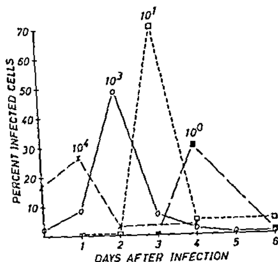


Fig 3

Variation in percentage of infected cells with time and inocula The inoculum used is given over each peak

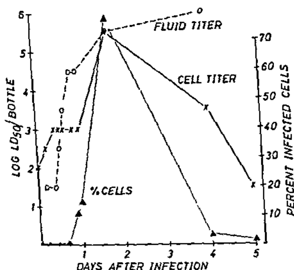


Fig 4

Detailed growth curve of 17D virus in CHL cells, showing changes in total intra- and extra cellular virus titre and percentage of infected cells Inoculum was $10^{3.5}$ LD_{50}

This correlation between infectivity titre and percentage of infected cells was not too apparent when the total intra- and extra-cellular infectivity was measured because the persistance of extra-cellular virus frequently masked changes in the intra-cellular infectivity

A more detailed growth curve can be seen in Fig 4 in which closely

spaced samples were taken for the first 24 hours after infection with $10^{3.5}$ LD_{50} 17D. For the first 14 to 15 hours the percentage of infected cells remained at a constant value of about 0.6 per cent, thereafter, this value increased sharply to about 70 per cent after 48 hours. This rise agreed closely with the appearance and increase of extra-cellular virus in the media. After 2 days CPE occurred, about 99 per cent of the cells sloughed off the glass and the percentage of infected cells remaining on the glass dropped to 1 or 2 per cent despite the presence of considerable infectious virus in the medium. The intra-cellular titre increased slightly the first few hours after infection and then levelled off during the period of initial virus liberation. The peak titre occurred about 48 hours after infection, after which the infectivity dropped sharply as more cells came off the glass. If the medium was left unchanged the number of cells remaining on the glass and the proportion of infected cells remained low and fairly constant for at least two weeks. If, however, the cells were washed and fresh media added, the proportion of infected cells increased from 1 per cent to 10 per cent about 3 days after the new medium was added. After 8 days the number of cells remaining on the glass and the virus titre became too small to measure. When the cells remaining after CPE were challenged with 10^{4} LD_{50} additional virus and fresh media added, the percentage of infected cells rose to about 40 per cent within 48 hours. Apparently, whatever resistance these cells had could be overcome if the cells were exposed to a high enough virus concentration.

TABLE 1

Comparison of Infection Produced by Crude 17D Virus Pool and Washed Virus

Type	Inoculum: Dilution	Inoculum: Titer LD_{50}/ml	Percentage of infected cells after 24 hours	Virus titer in total cell suspension
Crude virus	10^{0}	10^{4}	3.6	$10^{2.5}$
	10^{-1}	10^{3}	30.0	10^{3}
	10^{-2}	10	2.6	10^{3}
	10^{-3}	10^{1}	0.05	$10^{1.5}$
Washed virus	10^{0}	10^{5}	100.0	10^{4}
	10^{-1}	10^{4}	47.0	$10^{4.5}$
	10^{-2}	10^{3}	8.6	10^{4}
	10^{-3}	10^{2}	0.24	$10^{1.5}$

Interfering substance.—*Porterfield* (1959) reported that growth of 17D was inhibited by interferon produced by influenza virus. Experiments in our laboratory suggested that a viral interfering substance also accumulated in cell cultures infected with 17D. This interference became apparent when undiluted virus pools were found to yield fewer infected cells than a 10^{-1} dilution of the same pool. This effect is demonstrated in Table 1, in which various dilutions of a crude virus pre-

paration was used to infect CHL cells Crude virus, here, refers to the infectious culture medium which was normally used as a virus pool Twenty-four hours after infection only 3 6 per cent of the cells were infected in the cultures treated with undiluted virus, whereas 30 per per cent infected cells were found in cultures treated with a 10^{2} dilution of this pool The virus was centrifuged out of this pool at 100,000 X g for 1 hour in a Spinco preparative centrifuge, resuspended in PBS and clearified by centrifuging at 1,000 X g for 15 minutes in a horizontal head centrifuge This preparation was called "washed virus" An inverse relationship was obtained between dilution of washed virus and percentage of infected cells, with as high as 100 per cent of the cells infected by 24 hours when the highest concentration of virus was used This value was never obtained with crude virus pools

These data suggest that a substance was present in the crude pool that interfered with either the initial adsorption of the virus by cells or virus synthesis This activity was most apparent in infected cultures after the appearance of CPE If cells were infected with undiluted pools prepared from cultures before CPE occurred, no interference was observed even when the infectivity titre was as high or higher than the cultures after CPE Also suggestive was the fact that if undiluted crude virus pools was added to cell cultures already infected for 24 hours with 17D, virus production and CPE was greatly reduced The inhibitor was either present at relatively low concentrations or active only in relatively high concentrations since a 10^{1} dilution of the crude pool was always sufficient to eliminate its effect It could not be associated with the virus particle itself since washed virus did not show interference at a higher concentration of virus than was present in the crude pool It is true that heat inactivated 17D (60° C for 30 minutes) interfered with the infectivity of active virus but only when the ratio of inactive to active virus was 10 1 and only at high concentrations (an equivalent infectivity of 10^{4} LD_{50} inactive virus to 10^{3} LD_{50} active virus) If the ratio or the relative concentration of virus was reduced, interference was no longer obtained

The effect of cell concentration vs virus concentration—The optimal ratio of virus to cell was examined to see if variation in population density of cells affected the yield of virus Varying concentrations of virus and cells were mixed in Wasserman tubes and the cells were allowed to settle and attach to the glass After 3 days incubation the cultures were frozen and thawed once, pooled and titrated The results shown in Fig 5 suggest a definite optimum yield of virus at a cell concentration of 10^{5} CHL cells per tube regardless of the virus inoculum over a 4 log range Higher cell concentrations produced crowded conditions on the glass and quickly exhausted the media which would limit virus as well as cell growth Concentrations of cells lower than 10^{4} per tube gave lower yield of virus, perhaps because of the lower probability of virus cell contact or because the cells could not condition the media

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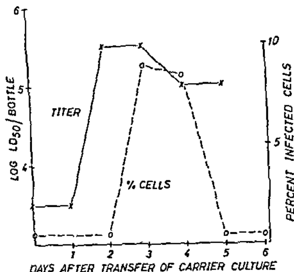


Fig 6

Typical growth curve of 17D virus in CHL carrier culture following passage of about 5×10^{5} cells/bottle The values represent the combined intra and extra cellular titre The percentage of infected cells are also given

was seldom above 10 per cent The small percentage of infected cells may explain why CPE was not detected After the 4th day the percentage of infected cells dropped once more to 1 per cent or less and remained at this low value, even though the cell number in the culture was still high and no significant CPE was noticeable Attempts to infect these cells with a challenging dose of 10^{4} LD_{50} virus did not significantly increase the virus yield or percentage of infected cells over that observed in the untreated carrier culture The apparent resistance of these cells was decreased by incubating the carrier culture at either 33° C or 40° C By the 7th day of incubation the percentage of infected cells was a high as 70 per cent at 33° C and 30 per cent at 40° C, while cultures at 37° C had only 2 per cent infected cells The cells did not grow as well at 40° C as they did at 37° C, but they grew quite well at 33° C although at a much slower rate than those at higher temperatures

The effect of various sera on the spread of infection—Increasing the concentration of horse serum in the medium to 20 per cent reduced the CPE produced by 17D virus This observation as well as the effect of other sera on the spread of infection was examined by measuring the percentage of infected cells 24 hours after initial infection (Table 2) The cells were grown in the lactalbumin hydrolysate medium containing 5 per cent horse serum until a confluent monolayer was obtained They were infected with $10^{2.5}$ LD_{50} for one hour, after which the cell layers were washed and media containing different sera were added The cells frequently showed morphological changes when a se-

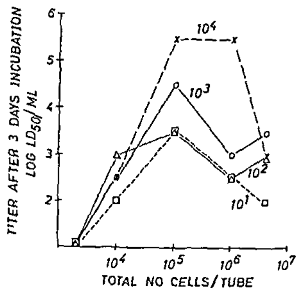


Fig 5

Effect of variation in cell concentration and virus inocula on viral yield after 3 days incubation The cells and virus were added to Wasserman tubes simultaneously in a total volume of 2 ml media The tubes were incubated at an angle for 3 days at 37° C, frozen and thawed once and titrated The inocula (10^1, 10^2, 10^3, and 10^4 LD_{50}) are indicated at the end of the curves

and grow well below this concentration Similar experiments using HeLa cells infected with 17D also gave the highest yield of virus at a cell concentration of 10^5 cells per tube These results agree with those of *Lund* (1961) who also found that cell population densities, as well as the age of the cell culture influenced the resulting infection of HeLa and Detroit-6 cells with polio virus, and suggest that population density may be an important factor in virus growth as it is with cell nutrition (*Eagle & Piez* 1962)

Carrier cultures—During the second passage of 17D in CHL cells some heavily infected cultures showed a strong CPE and then, within 2 or 3 days, the cells remaining on the glass grew out to form a normal looking monolayer These cells were passed once in media containing 10^3 LD_{50} 17D/ml The cells grew normally and did not show CPE in spite of the constant presence of virus in the media All subsequent passages were made without the addition of virus to the media These cells were maintained for over a year and were always morphologically indistinguishable from uninfected CHL cells despite the constant presence and growth of virus The growth curve of the virus in the carrier culture is shown in Fig 6 along with the change in the percentage of infected cells after the cells has been passed at a concentration of 5×10^5 cells per bottle The growth curve closely resembled that obtained from an initially infected cell culture although obvious CPE from virus growth could not be detected in the carrier culture The maximum proportion of infected cells was reached between the 3rd and the 4th day after passage (which also was the time of peak cell growth), but this value

of extra cellular virus, but the percentage of infected cells averaged about 0.7 per cent, which was similar to the value obtained from infected cultures containing immune human serum. *Porterfield & Rowe* (1960) found that the α lipoprotein fraction of human serum inhibited the haemagglutination of goose-red blood cells by Yellow Fever virus. The data presented here suggest that human serum may interfer also with virus adsorption onto cells.

DISCUSSION

The percentage of infected cells has become a sensitive parameter for studying cell-virus interactions and factors which influence this interaction. The method employed here in determining the percentage of infected cells permitted single, infected cells to come in direct contact with a large susceptible population so that liberated virus had a high probability of infecting other cells. Thus, a single infected cell would give rise to an infection detectable by CPE or by inoculation into the brains of mice. Because the volume of media overlaying the cell monolayer on the glass was high in this system (2 ml media per Wasserman tube), there was only a small probability for free virus that may have been present in the infected cell suspension to come in contact and infect the cells growing in the tube. Indeed, this procedure was about 2 log less sensitive than mouse titration for measuring virus suspensions, whereas the tube titration of infected cell suspensions was about one log more sensitive than mouse inoculations. Therefore, it is doubtful that any free virus which may have been present in the cell suspensions played a significant part in these titrations, except possibly when the percentage of infected cells was very low, less than 1 per cent.

Under the conditions used in these experiments, 100 per cent of the cells could not be infected after 1 hour adsorption with the initial inoculum of 17D, even when washed virus was used to eliminate the interference effect. The number of cells infected with an inoculum of 10^4 to 10^6 LD_{50} tended to be constant at about 10^4 cells, or approximately 10 to 20 per cent of the cell population. When an inoculum of 10^3 LD_{50} was used, approximately 1 to 0.5 per cent of the cells were infected. Apparently, 10^4 LD_{50} represented an inoculum beyond which no further increase in the percentage of infected cells resulted. Despite the fact that no more than 10 to 20 per cent of the cells *could be initially infected* with a high inoculum, virtually all of the cells became infected during the second cycle of virus growth. This marked increase was probably due to the continual release of virus in the medium after the first growth cycle and perhaps to the more efficient spread of virus from an infected cell to surrounding and closely associated susceptible cells. The possibility also exists, but as yet cannot be proven, that the CHL cells were susceptible to virus infection only at certain stages in their growth cycle, which might explain why only a small percentage

rum different from that in which they had been grown was suddenly introduced However, these morphological changes had no apparent influence on the virus yield The high population density used in these experiments (about 3 to 4 × 10^6 cells/bottle), helped to buffer the cells against radical changes in the environment, even incubation in a medium without any serum left most of the cells on the glass after a period of 24 hours, although the cells did not look healthy.

TABLE 2

Effect of Various Sera on Percentage of Infected Cells, Values Determined 24 Hours after Infection with $10^{2.5}$ LD_{50} 17D Virus

Serum	No cells bottle	*Total* cell titer	*Total* fluid titer	Percent infected cells
0 time	2.5 × 10^6	10^0	—	1.9
No serum	2.0 × 10^6	$10^{2.5}$	10^3	3.2
5 % horse	1.8 × 10^6	$10^{3.5}$	$10^{4.5}$	8.0
10 % horse	3.7 × 10^6	10^4	$10^{5.5}$	11.0
20 % horse*	2.6 × 10^6	$10^{3.5}$	$10^{5.5}$	20.0
10 % calf	5.6 × 10^6	$10^{4.5}$	10^6	3.2
10 % guinea pig	3.0 × 10^6	$10^{3.5}$	$10^{5.5}$	3.2
10 % mouse	5.2 × 10^6	$10^{4.5}$	10^5	19.0
10 % immune mouse	5.0 × 10^6	10^4	10^4	1.3
10 % human	4.3 × 10^6	10^4	10^5	0.7
10 % immune human	3.3 × 10^6	$10^{3.5}$	$<10^{0.5}$	0.5

* CPE greatly reduced at this concentration of horse serum

Table 2 shows that increasing the concentration of horse serum in the medium increased the percentage of infected cells and the amount of virus produced however, as mentioned previously, when 20 per cent horse serum was used the CPE, which generally occurred between the 2nd and 3rd day, was greatly reduced despite the excellent yield of virus demonstrated and the relatively high percentage of infected cells

10 per cent mouse serum appeared to be even better than the corresponding amount of horse, calf and guinea pig serum in promoting the spread of infection The serum from mice immunized against 17D by inoculating approximately 10^3 LD_{50} of live virus intraperitoneally reduced the spread of infection and virus yield, although an appreciable amount of virus was still present in the fluid and cell suspension This serum has very little neutralizing activity against 17D and yet mice immunized in this manner could survive an intra cerebral challenge of 10^3 LD_{50}

The 10 per cent anti-17D human serum, which was capable of neutralizing 10^3 LD_{50} of virus in one hour at 37° C, did not interfere with intracellular virus growth, but 24 hours after infection, no extra-cellular virus was detected and the percentage of infected cells was 0.5 per cent, which corresponded closely with the zero-time value When human sera from 4 individuals not immunized against 17D were tested, there was no interference with intra-cellular growth or neutralization

cell population These carrier cells also differed from the cells surviving after CPE in an initially infected culture in that they were not readily infected with a challenging dose of virus and were able to grow to large populations in a culture whereas cultures of cells surviving CPE usually died out within a week or two following CPE

... [illegible] clear that a population of cells either directly

can influence the development of a virus within itself, but certainly such a cell can influence the spread and subsequent growth of the virus in surrounding cells It is believed that studying changes in the percentage of infected cells may yield important information in this field of cell virus interaction

SUMMARY

The growth of Yellow Fever virus, strain 17D, was studied in Chang's human liver cells by conventional titration in mice and by a method which estimated the percentage of infected cells in the cell population

The initial growth cycle lasted 14 to 15 hours after infection and the virus was liberated for at least 10 hours thereafter The percentage of infected cells rose sharply until CPE occurred and most of the cells sloughed off the glass Of those cells remaining on the glass after CPE about 1 per cent or less were infected in spite of the fact that there was a high concentration of virus in the medium

Carrier cultures were established and shown to contain between 1 per cent to 10 per cent infected cells These cells proved to be highly resistant to challenging doses of virus

Evidence was presented that a substance(s) produced by infected cells limited the spread of virus in the cell population Non immune human serum limited the spread of infection also, probably by interfering with adsorption of the virus

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of cells can be initially infected in a randomly growing cell population

17D virus was first liberated about 14 hours after the initial infection This time may then be considered as that required for one growth cycle, although it was apparent from the results with human serum that the initially infected cells continued to secrete virus for at least 10 hours after the virus first appeared in the medium

After the initial CPE and the sloughing off of dead cells, approximately 1 per cent of the cells remaining on the glass were infected, the remainder maintained themselves uninfected in a medium containing on the average 10^3 LD_{50} virus/ml When fresh medium was added to such cultures the percentage of infected cells rose to about 10 per cent, but this occurred only after 3 days incubation in the new medium During that time there was no appreciable increase in cell number It would appear to take at least 2 to 3 days, therefore, for an interfering substance present in or on the cells to be either diluted out or destroyed

The data presented in this report suggests that a substance(s) was produced by 17D Yellow Fever virus infected cells that interfered with either adsorption of the virus onto cells or intra-cellular development of the virus, or perhaps both The relationship of this substance to interferon, which is able to interfere with the multiplication of 17D virus, has not been determined as yet, although *Hitchcock & Porterfield* (1961) reported that a Group (A) Arbovirus, O'nyong-nyong, produced interferon in infected mouse brain The interference with adsorption of the virus onto susceptible cells exhibited by human serum was different in action from interferon because the serum had no influence on intra-cellular growth The possibility exists that this serum interfering effect may be related to the Yellow Fever haemagglutinin-inhibition of the α lipoprotein fraction of human serum

The cells in the 17D-CHL carrier cultures appeared to have reached some equilibrium with the virus in which only a small number of cells became susceptible to infection at any one time It is doubtful that this equilibrium involved a genotypic change in resistance to 17D infection because the percentage of cells infected could be greatly increased by changing the incubation temperature This carrier system was possible only because the majority of the cells in the population somehow maintained their resistance to 17D, while only a few cells somehow lost this resistance and became infected The nature of this resistance and its relationship to the interfering substance found in initially infected cultures is not known They differ in one obvious respect, however When 17D was used to infect susceptible cultures of CHL cells the interfering substance was not detected until most of the cells had been destroyed by the virus *Wagner* (1963) showed that yields of interferon were greater from cells undergoing CPE than from those showing no discernible CPE following growth of EEE In the carrier cultures the interference substances were present early enough to prevent the spread of infection to a large number of cells and the massive destruction of the

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STUDIES ON THE ANTIGENIC STRUCTURE OF MYCOBACTERIA

Report 1

By

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Received 24 1 64

The classification of mycobacteria has mainly been based on the morphology of the colonies and the virulence to laboratory animals Within human bacteriology we formerly reckoned with four groups, *viz*, *typus humanus* t *bovinus* t *gallinaceus* and the large group of acid fast saprophytes which mainly caused differential diagnostic difficulties on demonstration of tubercle bacilli in gastric lavage and urine As far as these previous studies are concerned, reference is made to a series of papers from the State Serum Institute Copenhagen (4, 5) In paper No 5 the earlier literature from other countries has been referred in detail

By means of these rather simple methods, mycobacteria could be divided into the above mentioned four groups As our methods were not sufficiently sensitive for a final classification, a few atypical strains were entered between the human and the bovine types (5, 7, 8 9) Consequently since 1928 we have periodically tried to elaborate methods for a serological classification of mycobacteria which we considered the only one for a finer classification We have used complement fixation agglutination in absorbed sera precipitation, and agar diffusion methods with previous absorption With these methods we have been able to differentiate mycobacteria that differed markedly from each other but it proved impracticable to differentiate the human type from the bovine and hardly the avian type from the mammalian

After having produced purified tuberculin with a modified Seibert technique (6) we examined the possibility of using this method for classification by employing the intracutaneous tuberculin reaction on guinea pigs sensitized with the different mycobacteria (11)

We investigated this method because in veterinary pathology it had been employed for the differentiation of infections caused by the bovine and the avian types in cattle and swine This pioneer work was performed by *Oluf Bang* (1) and continued by V *Plum* (14)

Supported by grants from "*King Christian X s Foundation*" and *Miss P A Brandt s Foundation*

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Sensitization
intervals of six
second, third,
in the animals For this reason a spray with adrenalin is kept ready in case the
[illegible] severe

animals troublesome, spontaneous intestinal contractions are rarely observed)

After removal of as much blood as possible by cardiac puncture, about 30 cm of the distal end of the ileum is removed and placed in oxygenated Ringer's solution By means of a Pasteur pipette the visceral contents are washed out, and the gut is placed in a new container with oxygenated Ringer's solution Thereafter it is cut into suitable pieces, and a piece of fine sewing cotton is attached to each end in order to fix the gut in the Schultz-Dale apparatus This procedure should be carried out with the utmost care

We have been able to keep a gut for up to 150 minutes and have performed up to twenty experiments in the Schultz Dale apparatus without any effect upon the reactivity of the gut

In the Schultz Dale bath the contractions of the gut were recorded kymographically The response of the strips of intestine to a standard dose of 0 15 microgram of histamine acid phosphate calculated as histamine base was observed before and after each series of tests After having been thoroughly washed with oxygenated Ringer's solution the intestine is rendered refractory to the first antigen (1 ml of the sterile filtered medium), and when the refractory state has been attained the intestine is tested with the same dose of the second antigen to be tested In order to control whether a contraction after exposure to the last antigen is due to an antigen antibody reaction and not to a pseudo reaction it is ascertained whether the gut has become refractory to this antigen too Each experiment has been repeated at least twice

Strains of Mycobacteria Employed

E5	Eugonic human strain cultivated from sputum
Av 1	Avian strain cultivated at the State Serum Institute, Copenhagen
1500	Eugonic, smooth, orange pigmented, fast-growing strain cultivated twice from sputum at the University Institute of General Pathology, Copenhagen
5824	Moderate eugonic, non-pigmented saprophyte cultivated four times from sputum at the University Institute of General Pathology, Copenhagen
Bal 14	Myc balnei isolated from the cemented walls of a swimming pool, Örebro, Sweden
8768	Eugonic, smooth, orange-pigmented saprophyte cultivated once from sputum at the University Institute of General Pathology, Copenhagen
Ross	Moderate eugonic, smooth, yellow-pigmented strain (Myc kansasii) Received from Mitchison

Experimental Series 1

During our preliminary experiments on guinea-pigs sensitized with different antigens produced from a human strain (E5), an avian strain (Av 1), and a fast-growing saprophyte (1500), we found that the only

In the experiments reported here we examined five human, four bovine, and one avian strain as well as two saprophytes isolated from man and two saprophytes isolated from water

The experiments showed that human and bovine tuberculin presented no definite differences in the reactions on guinea-pigs infected with human and bovine strains On the other hand, there was a distinct difference between these two tuberculins and the avian and saprophytic tuberculins Further, there was also a distinct difference between the avian tuberculin and the various saprophytic tuberculins In every case the homologous tuberculin gave stronger reactions than the heterologous tuberculin

The saprophytes could roughly be divided into two groups, one comprising saprophytes isolated from man, the other comprising saprophytes isolated from water *Still*, there was also some difference between the various saprophytes within the two groups Here, too, as a rule the homologous tuberculin produces stronger reactions than the heterologous

Later this method of classification has been used by *Magnusson* (13) *Dragsted* (2), *Edwards & Palmer* (3), and many others have also used it in their investigations to find out which type of mycobacterium infects man, as well as in epidemiological studies This method has, however, the drawback that it yields only a quantitative and no qualitative difference An antigen scheme is therefore difficult to set up, nor can slighter antigenic differences be demonstrated

During the past year we have elaborated a method that yields a qualitative, specific result which makes it possible to elaborate an antigen scheme for mycobacteria

INVESTIGATIONS

For some time past we have used the Schultz Dale method for antigen analysis

TECHNIQUE

Production of antigens In this paper we shall only mention the antigen that we considered to be the most suitable in the preliminary investigations

When a culture of mycobacteria grows well on *I L T M* (*10*) (modified Lowenstein's medium) it is subcultivated on Sauton's medium as a film Then ten to twenty tuberculin flasks are inoculated with the primary Sauton culture In order that the culture may be serviceable it must cover the entire surface of the medium four weeks after the inoculation with a *thick film* The culture is then passed through filter paper and afterwards through a Berkefeld filter No 5 The pH value is adjusted to 7 7 2 with hydrochloric acid or sodium hydroxide Finally it is passed through a Jena glass filter G_3

It is then poured into small tubes which are used only once in order to prevent contamination The tubes are kept at +4° C and the antigen is stable for at least one year

In order to ascertain that no growth of other bacteria or mould has taken place during the cultivation of mycobacteria we control the sterility of the medium as well as of the film by cultivating on blood agar and in semi solid medium

TABLE 1

Animal	Antigen	Strength		Antigen	Strength		Antigen	Strength		Antigen	Strength	
No 470 I 5	Av 1	++++	1	Av 1	0	2	I 5	++++	3	F5	0	4
	1500	++++	1	1500	0	2	I 5	++++	3	I 5	0	4
	Bal 14	++++	1	Bal 14	0	2	I 5	++++	3	F5	0	4
No 471 I 5 + adjuvant	Av 1	+++	1	Av 1	0	2	I 5	++++	3	E5	0	4
	1500	++++	1	1500	0	2	I 5	++++	3	I 5	0	4
	I 5	++++	1	L5	0	2	1500	0	3			
No 472 1500	I 5	++++	1	I 5	0	2	1500	++++	3	1500	0	4
	Av 1	++++	1	Av 1	0	2	1500	++++	3	1500	0	4
	Bal 14	++	1	Av 1	++++	2	1500	++++	3	1500	0	4
	Av 1	++++	1	I 5	+++	2	1500	++++	3			
	I 5	++++	1	Av 1	0	2	1500	++++	3			
	1500	++++	1	1500	0	2	I 5	0	3			
No 473 1500 + adjuvant	I 5	0	1	1500	++	2						
	Av 1	0	1	1500	++	2						
	Bal 14	0	1	1500	++	2						

0 ++++ indicates the strength of the intestinal contraction The figures 1 2 3 4 after indication of the strength indicate the sequence of the addition of the antigens to the Schultz Dale bath after washing with Ringer's solution The first column gives the number of the experimental animal and the antigen with which it has been sensitized The following columns show the antigens employed They are all unheated sterile filtered Sauton's culture filtrates and in all the experiments 1 ml was used

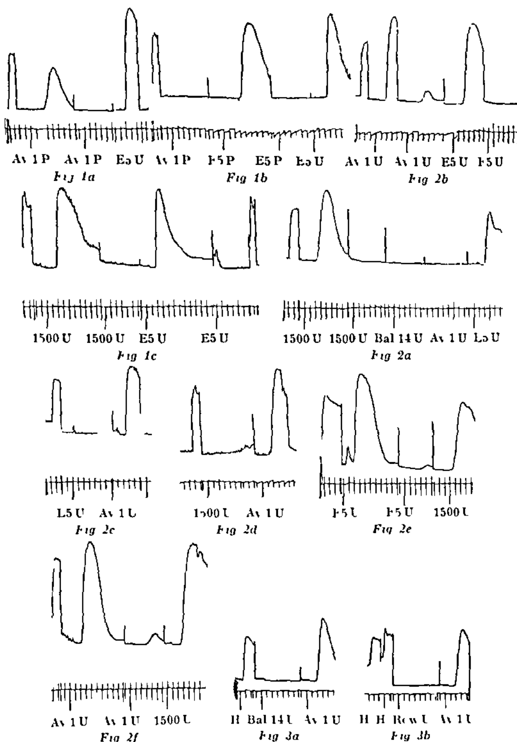


Fig 1a b and c Guinea pig No 129 sensitized with unheated E5 medium
H = histamine Av 1 P = PPD Av 1 U = unheated medium E5 P = PPD
E5 U = unheated medium 1500 P = PPD 1500 U = unheated medium
Fig 2a and b Guinea pig No 470 sensitized with unheated E5 medium
Fig 2c and d Guinea pig No 711 sensitized with unheated Av 1 medium
Fig 2e and f Guinea pig No 472 sensitized with unheated 1500 medium
Fig 3a and b Guinea pig No 710 sensitized with unheated Av 1 medium

The first contraction in Figs 1 and 2 represents a reaction to histamine as shown in Fig 3

TABLE 1

No 470 1 5	Av 1	++++	1	Av 1	0	2	1 5	++++	3	1 5	0	4
	1500	++++	1	1500	0	2	1 5	++++	3	1 5	0	4
	Bal 14	++++	1	Bal 14	0	2	1 5	++++	3	1 5	0	4
No 471 1 5 + adjuvant	Av 1	+++	1	Av 1	0	2	1 5	++++	3	1 5	0	4
	1500	++++	1	1500	0	2	1 5	++++	3	1 5	0	4
	1 5	++++	1	1 5	0	2	1500	0	3			
No 472 1500	1 5	++++	1	1 5	0	2	1500	++++	3	1500	0	4
	Av 1	++++	1	Av 1	0	2	1500	++++	3	1500	0	4
	Bal 14	++	1	Av 1	++++	2	1500	++++	3	1500	0	4
	Av 1	++++	1	1 5	+++	2	1500	++++	3			
	1 5	++++	1	Av 1	0	2	1500	++++	3			
	1500	++++	1	1500	0	2	1 5	0	3			
No 473 1500 + adjuvant	1 5	0	1	1500	++	2						
	Av 1	0	1	1500	++	2						
	Bal 14	0	1	1500	++	2						

0-++++ Indicates the strength of the intestinal contraction The figures 1 2 3, 4 after indication of the strength indicate the sequence of the addition of the antigens to the Schultz Dale bath after washing with Ringer's solution The first column gives the number of the experimental animal and the antigen with which it has been sensitized The following columns show the antigens employed They are all unheated sterile filtered Sauton's culture filtrates and in all the experiments 1 ml was used

antigen that produced a *specific reaction* was an unheated, freeze-dried, dialyzed filtrate of a Sauton culture Guinea-pigs sensitized with four subcutaneous injections of 5 mg of this antigen yielded in Schultz-Dale's bath a specific contraction of the gut to PPD tuberculin E5 after it had become refractory to avian PPD (cf Fig. 1 a)

After the gut had become refractory to PPD E5, a surprisingly strong contraction was elicited when filtrate of unheated E5 Sauton's culture was added (cf. Fig 1 b) Furthermore, it was seen that this filtrate of unheated Sauton's culture was the one most suitable for sensitization and for eliciting specific reactions (cf Fig 1 c)

Experimental Series 2

In this series of experiments, an unheated filtrate of an approximately four-weeks-old Sauton culture that covered the entire surface of the medium with a thick film was used as *antigen for sensitizing guinea-pigs* and eliciting the intestinal contraction

Guinea-pig No 470 was sensitized with four subcutaneous injections of 5 ml of medium E5 at intervals of one week Guinea-pig No 471 was sensitized in the same way with 2 5 ml of E5 medium in Freundt's adjuvant (without mycobacteria) Guinea-pig No 472 was sensitized with four subcutaneous injections of 5 ml of medium 1500, and guinea-pig No 473 was likewise sensitized with 2 5 ml of medium 1500 in Freundt's adjuvant The result of this appears from Fig 2 a, b, c, d, e, f, which illustrates the procedure of the experiment, as well as from Table 1, in which the total result is recorded This experiment shows that *addition of Freundt's adjuvant to the antigen used* (cf Nos 472 and 473) offers no advantage whatever

It is further apparent from this experiment that E5, Av 1, 1500, and Bal 14 possess a strong, common antigen (cf Fig 2 and Table 1) Moreover, it will be seen from Table 1 that both E5 and 1500 possess a distinct, *specific antigen*

The experiments with guinea-pig No 472 (cf Fig 2 f and Table 1) show simultaneously that there are more specific antigens than the three mentioned hitherto An analysis of these antigens cannot be performed before guinea-pigs have been sensitized with a filtrate of Sauton's culture of the other strains (Av 1 and Bal 14)

The experiment also shows that the specific reactions cannot be due to one antigen being more effective than the other Antigen E5 elicits a stronger reaction than does 1500 on Nos 470 and 471, while 1500 elicits a stronger reaction on No 472 than does E5 In other words, the reactions are qualitatively specific *when the gut has been made refractory to an antigen* (cf Fig 2)

Experimental Series 3

In order to examine the heat stability of the antigens in the sterile filtered Sauton's medium guinea pig No 499 was sensitized with three injections of unheated E5 Guinea pig No 495 was sensitized in the same way with the same filtrate *steamed for one hour and No 497* with the filtrate autoclaved for one hour

TABLE 2

	Autoclaved E5		Steamed E5		Unheated E5	
No 499 E5 unheated	0	1	++++	1	++++ ++	2 2
No 495 E5 steamed	+++	1	0 ++++ 0	2 1 2	0 ++++	2 1
No 497 E5 autoclaved	++++ 0 0	1 2 2	++++	1	0 ++	2 1
	Autoclaved Av 1		Steamed Av 1		Unheated Av 1	
No 711 Av 1 unheated	0	1	0	1	++++ ++++	2 2
No 707 Av 1 steamed	0	1	0 0 0	2 1 2	++++ ++++ ++++	3 2 1
No 709 Av 1 autoclaved	+ 0 0	1 2 2	0 +	2 1	+	1
	Autoclaved 1500		Steamed 1500		Unheated 1500	
No 05 1500 unheated	0	1	++	1	++++ ++	2 2
No 01 1500 steamed	++++	1	0 ++++ 0	2 1 2	0 ++++	2 1
No 03 1500 autoclaved	+++ 0 0	1 2 2	++++	1	++++	1

Explanation Cf Table 1

Similarly, three guinea pigs were sensitized with Av 1, and thre guinea-pigs with 1500 Eight to ten days after the last sensitizing in jection we examined the same antigens by means of the Schultz Dal method The result appears from Table 2

TABLE 3

	Autoclaved E5		Steamed E5		Unheated E5	
No 498 E5 unheated	++++ ++++	1 1	+ ++++	2 1	0 0 ++	3 2 2
No 494 E5 steamed	+++	1	0 0 ++++	2 2 1	0 +++ 0	3 1 2
No 496 E5 autoclaved	++++ 0 0	1 2 2	++++	1	0 ++++	2 1
	Autoclaved Av 1		**Steamed Av 1**		**Unheated Av 1**	
No 710 Av 1 unheated	0 0	2 1	0	1	++++ ++++ ++++	1 2 2
No 706 Av 1 steamed	0	1	0	1	+++ +++	2 2
No 708 Av 1 autoclaved	++++ 0 +++	2 2 1	++++ 0	1 2	0	1
	Autoclaved 1500		**Steamed 1500**		**Unheated 1500**	
No 704 1500 unheated	0	1	0 ++	2 1	++++ 0 ++++	1 2 2
No 500 1500 steamed	++++ +++	1 1	++++ 0 +	1 2 2	0 ++++ 0	2 1 2
No 702 1500 autoclaved	+ 0	1 2	0	1	+	1

Explanation Cf Table 1

TABLE 4

No 499 E5 unheated	Av 1	++++	1	F5	++++	2
	1500	++	1	F5	++++	2
No 495 F5 steamed	Av 1	0	1	F5	++++	2
	5824	0	1	F5	++++	2
No 497 E5 autoclaved	Av 1	0	1	E5	++++	2
	1500	0	1	F5	+++	2
No 711 Av 1 unheated	F5	0	1	Av 1	++++	2
	1500	0	1	Av 1	++++	2
	5824	0	1	Av 1	++++	2
No 707 Av 1 steamed	E5	0	1	Av 1	++++	2
	1500	0	1	Av 1	++++	2
No 709 Av 1 autoclaved	E5	0	1	Av 1	0	2
	1500	0	1	Av 1	0	2
No 705 1500 unheated	E5	0	1	1500	++++	2
	Av 1	0	1	1500	++++	2
No 701 1500 steamed	F5	0	1	1500	++++	2
	Av 1	0	1	1500	++++	2
	5824	0	1	1500	++++	2
No 703 1500 autoclaved	E5	0	1	1500	++++	2
	Av 1	0	1	1500	++++	2
	5824	0	1	1500	++++	2

Explanation Cf Table 1

TABLE 5

No 498 F5 unheated	Av 1	++++	1	E5	++++	2
	1500	++++	1	F5	++++	2
	Bal 14	++++	1	F5	++++	2
	5824	++++	1	F5	++++	2
	8 68	+++	1	F5	++++	2
	Row	++++	1	F5	++++	2
No 494 E5 steamed	Av 1	++	1	E5	+++	2
	1500	++	1	E5	+++	2
	Bal 14	++	1	F5	+++	2
	5824	+++	1	F5	++++	2
	8 68	+++	1	F5	++++	2
	Row	+++	1	F5	++++	2

Similarly, three guinea-pigs were sensitized with Av 1, and three guinea-pigs with 1500 Eight to ten days after the last sensitizing injection we examined the same antigens by means of the Schultz Dale method The result appears from Table 2

TABLE 3

	Autoclaved E5		Steamed E5		Unheated E5	
No 498 E5 unheated	++++ ++++	1 1	+ ++++	2 1	0 0 ++	3 2 2
No 494 E5 steamed	+++	1	0 0 ++++	2 2 1	0 +++ 0	3 1 2
No 496 E5 autoclaved	++++ 0 0	1 2 2	++++	1	0 ++++	2 1
	Autoclaved Av 1		**Steamed Av 1**		**Unheated Av 1**	
No 710 Av 1 unheated	0 0	2 1	0	1	++++ ++++ ++++	1 2 2
No 706 Av 1 steamed	0	1	0	1	+++ +++	2 2
No 708 Av 1 autoclaved	++++ 0 +++	2 2 1	++++ 0	1 2	0	1
	Autoclaved 1500		**Steamed 1500**		**Unheated 1500**	
No 704 1500 unheated	0	1	0 ++	2 1	++++ 0 ++++	1 2 2
No 500 1500 steamed	++++ +++	1 1	++++ 0 +	1 2 2	0 ++++ 0	2 1 2
No 702 1500 autoclaved	+ 0	1 2	0	1	+	1

Explanation Cf Table 1

had been sensitized with four injections The result is recorded in Table 3

The same eighteen guinea pigs have *also been employed for* a temporary, serological classification of the three strains used for the sensitization (E5, Av 1, and 1500)

Furthermore, we have examined strains 5824, Bal 14, 8768, and Row in order to ascertain the occurrence of a common antigen A more exact antigen analysis of these strains can only be made after having sensitized guinea pigs with them Tables 4 and 5, and Figure 3a, b *illustrate these extensive experiments*

From the antigen analysis listed in Tables 4 and 5 it will be seen that all the strains tested possess a common antigen which we have provisionally designated as X

It is of interest to note that the effect of this common antigen is only seen in guinea pigs Nos 499 and 498, both sensitized with unheated E5 and in one of the two guinea pigs, No 494, sensitized with steamed E5 In the two guinea pigs sensitized with autoclaved E5, Nos 497 and 496 only reactions are seen which are specific to the homologous antigen All the other guinea pigs sensitized with Av 1 and 1500 showed reactions that likewise are specific to the homologous antigen only

The presence of a common antigen does not, however, affect the investigations carried out with the Schultz Dale method Consequently, in further investigations we shall use the unheated antigen for sensitization as well as for eliciting intestinal contractions

On the basis of our previous experiments, a provisional antigen scheme for the strains tested is recorded in Table 6

TABLE 6

	Common antigen	Specific antigen
E5	×	1
Av 1	×	2
1500	×	3
Bal 14	×	?
5824	×	?
6868	×	?
Row	×	?

Explanation Cf Table 1

With regard to the last four strains mentioned in Table 6 we have not been able to demonstrate a specific antigen in this investigation Such a demonstration is only possible with the method *employed after* sensitization of guinea pigs with each of the four strains, as was the case with strains E5, Av 1, and 1500 These investigations are being carried out using a greater number of strains

TABLE 5 (cont)

No 496 E5 autoclaved	Av 1	0	1	E5	+++	2
	1500	0	1	E5	+++	2
	Bal 14	0	1	E5	++	2
	5824	0	1	E5	+++	2
	8768	0	1	E5	++	2
	Row	0	1	E5	+++	2
No 710 Av 1 unheated	E5	0	1	Av 1	++++	2
	1500	0	1	Av 1	++++	2
	Bal 14	0	1	Av 1	++++	2
	5824	0	1	Av 1	+++	2
	8768	0	1	Av 1	++++	2
	Row	0	1	Av 1	++++	2
No 706 Av 1 steamed	E5	0	1	Av 1	++++	2
	1500	0	1	Av 1	++++	2
	Bal 14	0	1	Av 1	++++	2
	5824	0	1	Av 1	++++	2
	8768	0	1	Av 1	++++	2
	Row	0	1	Av 1	++++	2
No 708 Av 1 autoclaved	E5	0	1	Av 1	++++	2
	1500	0	1	Av 1	+++	2
	Bal 14	0	1	Av 1	+++	2
	5824	0	1	Av 1	++	2
	8768	0	1	Av 1	+++	2
	Row	0	1	Av 1	+++	2
No 704 1500 unheated	E5	0	1	1500	+++	2
	Av 1	0	1	1500	++++	2
	Bal 14	0	1	1500	+++	2
	5824	0	1	1500	+++	2
	8768	0	1	1500	+++	2
	Row	0	1	1500	++++	2
No 500 1500 steamed	E5	0	1	1500	++++	2
	Av 1	0	1	1500	++++	2
	Bal 14	0	1	1500	++++	2
	5824	0	1	1500	++++	2
	8768	++	1	1500	+++	2
	Row	0	1	1500	+++	2
No 702 1500 autoclaved	E5	0	1	1500	+	2
	Av 1	0	1	1500	+	2

Explanation Cf Table 1

From this table it is seen that the unheated medium used for sensitization and eliciting of intestinal contractions affords the most reliable results This applies to the avian strain in particular

The same experiment has been carried out on guinea pigs, which

Thus, the technique for antigen analysis mentioned in this paper requires a great deal of experience The results obtained so far have, however, been so encouraging that we have started testing numerous, *different strains*

There is a phenomenon that may cause a wrong interpretation Phage type determination of mycobacteria is increasingly being employed *Russell et al* (15) have shown that lysogenic cultures of mycobacteria occur and that this lysogenicity alters the phage types *If such lysogenic* mycobacteria appear in the material to be examined, the culture will possess an antigen which does not belong to the mycobacterium concerned This source of error may, however, occur in several other serological antigen studies, *especially if the agar-diffusion* technique is employed

SUMMARY

By sensitizing guinea pigs with unheated, sterile filtrate of Sauton cultures of mycobacteria, it is practicable with the Schultz-Dale technique to demonstrate a specific antigen for each of the three strains examined (i e human strain E5, Av 1, and a fast-growing saprophyte 1500) In addition to the type specific antigen a common antigen has been found, which has also been demonstrated in four other mycobacterial strains

In order to be able to demonstrate the specific antigen, guinea pigs *must be sensitized with each of the strains to be examined, and cross-*antigenic reactions should be carried out in the Schultz Dale apparatus These experiments will be published later

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Experimental Series 4

In order to avoid using the Schultz-Dale technique, which requires a great deal of experience, we have investigated whether it was possible to demonstrate the antibodies in the sensitized guinea-pigs by means of the agar-gel-diffusion method and agglutination

Blood samples were taken from the guinea pigs prior to investigations in the Schultz-Dale apparatus In none of the sera were we able to demonstrate agglutinin Conversely, on employment of the agar-diffusion technique modified by *Klaus Jensen* (12) it was practicable in *some of* the guinea-pigs sensitized with unheated E5 to demonstrate a well-defined line for the homologous antigen exclusively We are, however, now immunizing guinea-pigs and rabbits more intensively by increasing the number of injections of antigen, in order, if possible, to obtain better results with the agar-diffusion technique

DISCUSSION

The method so far most employed for serological classification of mycobacteria is based on a quantitative difference in intracutaneous tuberculin reactions in homologously and heterologically sensitized guinea pigs Of course, a more thorough classification cannot be made with this technique

When using the Schultz-Dale technique and unheated, sterile filtered Sauton's culture as antigen, we succeeded in demonstrating specific antigens in the three strains, E5, Av 1, and 1500—in addition to a common antigen The chief requirement for the carrying out of this antigen analysis is that the cultures used are free from contamination with bacteria or mould Before harvesting the cultures, a sterile control must be made from all the flasks As both mould and bacteria can grow in the culture film without contamination being observed, a piece of the film must be tested by the sterile control The sterile filtered medium can be stored at $+4°$ C for a long time without loosing its strength

The method employed has, however, the drawback that wide experience is necessary in order to be able to obtain growth of the dysgonic mycobacteria on the surface of Sauton's medium Several subcultivations are necessary before obtaining a culture that within four to six weeks forms a thick film on the surface of the medium

The culture filtrate is adjusted to a pH of 7 7 2 If the medium is too alkaline, pseudo-reactions arise when the Schultz-Dale method is employed, and this may be rather inconvenient if the gut has to be made refractory If the medium is too acid the contractions of the gut will become weak or even fail to appear In a later paper one of the present authors, L Lundberg, will describe the importance of the concentration of hydrogen ions in the Schultz-Dale bath

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STUDIES ON THE ANTIGEN DEMONSTRATED BY MALIS IN SERUM FROM SCHIZOPHRENIC PATIENTS[1]

By

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patients with chronic schizophrenia, they demonstrated an antigen not found in patients with other diseases or in normal persons The antigen concerned could be demonstrated by a number of *different methods*, *e g* two *in vitro* (agglutination and complement fixation) and one *in vivo* method (anaphylaxis)

As a specific antigen was demonstrated in the blood of schizophrenic patients on employment of all three methods the hypothesis was advanced that schizophrenia is due to a virus and that the antigen is the virus itself or is liberated from it

Another study by *Haddad & Rabe* (1961) has confirmed that this antigen can be demonstrated in the serum from schizophrenic patients by the anaphylactic test

In the present study we tried to demonstrate this antigen, and having previously used the anaphylactic method for antigen analysis, we chose this as our experimental method Apart from the technique used by *Malis et al* we employed the more sensitive and less subjective *Schultz-Dale* method

In their four anaphylactic experiments *Malis et al* used the following technique

In the first series they sensitized 8 guinea pigs by one subcutaneous injection of 0 1 ml serum from schizophrenic patients

Desensitization was performed 21 days later First 0 5 ml normal human serum was injected subcutaneously A second *intracardial* injection of normal serum 24 hours later gave no reaction which was evidence of complete desensitization (dosage not stated)

Twenty four hours after the desensitization 0 1 *ml of serum from*

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[1] The present work has been carried out with the aid of grants from *King Christian X s Foundation* and "*Miss P A Brandt s Foundation*

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TABLE 1
Patient Material

No	Diagnosis	Age	Duration of disease years	No of years in hospital	Main symptoms	Treatment with psychopharmaceutics	Somatic diseases
1	Schiz	56	21	17	Autism delusions halluc	Medication discont 2 weeks before test	0
2		64	30	35			0
3		61	20	11	Severe autism marked halluc		Chron otitis media
4		59	34	29			0
5	"	59	25	25	Autism delusion halluc		0
6	"	53	31	31			0
7	"	56	34	34			0
8	Schiz	76	34	34	Autism delusions halluc	None	Anal prolapse
9		82	53	53	"		0
10	"	76	22	14	"		Myxedema
11	"	56	31	31	" "	"	0
12		82	35	25			Heart disease
16	Psychopath drug addict	41		1/4	Depression	Chlorpr at test	Pyel nephritis
17	Manic depressive psych alcohol abuse	62		1/2	Depressive dysphoric phases		0
18	Manic depressive psych	40		1/12		"	0
19		65	30	14	Depressive and manic phases		0

schizophrenic patients was injected into the hearts of all the guinea-pigs. Marked signs of anaphylaxis were observed in 7 of the 8 guinea-pigs. Four died in shock.

In the second series 15 guinea-pigs were sensitized with 0.1 ml serum from schizophrenic patients. Twenty-one days later they were desensitized by one subcutaneous injection of 0.5 ml of normal serum. Twenty-four hours later 0.1 ml serum from schizophrenic patients was injected into the heart.

In 14 of the 15 guinea-pigs they observed anaphylactic symptoms of various degrees of severity. Three died in shock.

In a control experiment 6 guinea-pigs were sensitized in the same way, but desentitized with serum from schizophrenic patients. As a challenge dose they injected intravenously 0.1 ml serum from schizophrenic patients. No anaphylactic reactions were observed.

In another control experiment 6 guinea-pigs were sensitized and desensitized by a subcutaneous injection of normal serum. Intracardial injection of serum from schizophrenic patients did not give rise to anaphylactic reactions.

In a third and fourth series *Malis et al.* injected the desensitizing and challenge dose intravenously, and the challenge dose, 0.15 ml, was injected 2 hours after the desensitization.

These two experimental series gave the same result as the previous ones.

It may be mentioned that *Malis et al.* used only one desensitizing injection, while *Haddad & Rabe,* who confirmed *Malis et al.*'s findings, had greater difficulties in desensitizing their experimental animals.

They report: "Thus, a single half ml subcutaneous desensitizing injection did not produce adequate desensitization of our animals as it had of those of *Malis*".

Accordingly, they first administered 0.5 ml subcutaneously, continuing by intracardial injections until the desensitization to normal serum was complete.

Animals that did not meet with the criterion after the fourth intracardial desensitizing injection were discarded. Like *Malis et al.*, they observed anaphylactic reactions in the desensitized guinea pigs upon injection of serum from schizophrenic patients.

PRESENT EXPERIMENTS

The patients from which the blood samples were taken are listed in Table 1.

The schizophrenic patients Nos. 1 ... 26 and 32, 33 were suffering from severe chronic schizophrenic psychosis of more than 10 years' duration and had been in hospital throughout, or almost throughout, the entire duration of their illness. All had active symptoms in the form of hallucinations and delusions, pronounced autism, besides a number of secondary schizophrenic symptoms.

All the patients had been treated for several years with various psych pharmaceutics mainly chlorpromazine which had caused sedation and improved behaviour but not eliminated other symptoms

Case 31 was suffering from severe paranoid schizophrenia presumably of many years standing

Cases 8 12 also had severe chronic schizophrenic psychoses but had not received psych pharmaceutics partly because they had not been particularly worried by their symptoms and had not been disturbed or aggressive and partly because they were

sample was obtained but later disappeared on chlorpromazine and chlorp... medication

Cases 16–19 were not schizophrenics Three had manic depressive psychosis one had been in hospital continuously for 14 years while the other two had been admitted at intervals in manic or depressive phases The fourth patient (Case 16) had been admitted because of drug abuse but was not psychotic

Normal human serum was taken from 4 members of the staff (and in the last experiment from 4 members of the staff of the University Hospital)

Experimental Series 1

Technique

The sera used in the experiment were prepared from blood samples taken on an empty stomach" and throughout the experiment the samples were stored at —20° C.

ne medication

and Case 31)

into the distal part of the ear where the veins though small are distinctly visible They can be made more visible by rubbing the ear with xylene Only white guinea pigs were used The needles used were Acufirm 0 40 × 21 It is easy to ascertain that the injection has been intravenous as otherwise a bulge will soon be seen on the ear and the resistance to the injection will be great

Experiment 1

In this experiment our procedure of sensitization was not exactly like Malis et al s as we administered 0 5 ml instead of 0 1 ml of patient serum

Our preliminary studies showed that unlike Malis et al we could not desensitize the guinea pigs by 1 or 2 injections of normal human serum

Table 2 shows for instance that guinea pig F 30 injected subcutaneously with 0 5 ml normal human serum died in shock following intravenous injection of 0 2 ml normal serum 24 hours later The same table shows that even after two subcutaneous injections of 0 5 ml normal serum at intervals of 24 hours intravenous injection of 0 1 ml normal serum 24 hours later gave rise to serious anaphylactic reactions in 3 out of 6 guinea pigs and mild reactions in the remaining 3 Like *Haddad & Rabe* therefore we had to use a desensitization more prolonged than that employed by Malis et al (cf Tables 2 3 and 4) As is apparent from Table 2 4 of the 5 guinea pigs that were injected intravenously with 0 1 ml of schizophrenic serum after desensitization with normal

TABLE 1 (*cont*)

No	Diagnosis	Age	Duration of disease (years)	No of years in hospital	Main symptoms	Treatment with psychopharmaceutics	Somatic diseases
22	Schiz	53	10	10	Autism, delusions, halluc	*Medication discont.* 2 weeks before test	0
23	,,	56	25	17	,, ,, ,,	,, ,,	0
24	,,	24	10	9	,, ,, ,,	Chlorpr at test	0
25	,,	70	37	37	,, ,, ,,	Reserpine at test	0
26	,,	67	22	19	,, ,, ,,	Chlorpr at test	0
31	Acute schiz	19	1/6	1/6	Anxiety, halluc on all senses, splitting of thoughts, delusions, no insight	Chlorpr. and chlorprothixen at test	0
32	Schiz	56	26	26	Autism, delusions	Medication discont. 2 weeks before test	History of pulm tub
33	Schiz	58	33	28	Autism, delusions, halluc	*Medication discont* 2 weeks before test	*History of uterine carc.*
34	Schiz	54	4	1½	Delusions	Medication discont 2 weeks before test	0

l pharma

... years standing

Cases 8 12 also had sc psychopharmaceutics pa symptoms and had not ... rather old

Case 31 had one of her present adm involving anxiety, delusions and disti sample was obtaine... medication

Cases 16–19 were not schizophrenics Three had manic depressive psychosis, one had been in hospital continuously for 14 years, while the other two had been admitted at intervals in manic or depressive phases The fourth patient (Case 16) had been admitted because of drug abuse, but was not psychotic

Normal human serum was taken from 4 members of the staff (and in the last experiment from 4 members of the staff of the University Hospital)

Experimental Series A

Technique

The sera used in the experiment were prepared from blood samples taken on an empty stomach" and throughout the experiment the samples were stored at —20° C. In cases where the serum was taken from patients in chlorpromazine medication, this medication had been discontinued (except in Cases 24-26 incl and Case 31) about 2 weeks before the removal of the blood sample

The shock experiments were carried out by intravenous injections into the distal part of the ear where the veins though small are distinctly visible They can be made more visible by rubbing the ear with xylene Only white guinea pigs were used The needles used were Acufirm 0 40 × 21 It is easy to ascertain that the injection has been intravenous as otherwise a bulge will soon be seen on the ear, and the resistance to the injection will be great

Experiment 1

In this experiment our procedure of sensitization was not exactly like *Valis et al* s, as we administered 0 5 ml instead of 0 1 ml of patient serum

Our preliminary studies showed that unlike *Valis et al* we could not desensitize the guinea-pigs by 1 or 2 injections of normal human serum

Table 2 shows, for instance, that guinea-pig F 30, injected subcutaneously with 0 5 ml normal human serum, died in shock following intravenous injection of 0 2 ml normal serum 24 hours later The same table shows that even after two subcutaneous injections of 0 5 ml normal serum at intervals of 24 hours, intravenous injection of 0 1 ml normal serum 24 hours later gave rise to serious anaphylactic reactions in 3 out of 6 guinea pigs, and mild reactions in the remaining 3 Like *Haddad & Rabe*, therefore, we had to use a desensitization more prolonged than that employed by *Valis et al* (cf Tables 2, 3, and 4) As is apparent from Table 2 4 of the 5 guinea-pigs that were injected intravenously with 0 1 ml of schizophrenic serum after desensitization with normal

human serum developed mild and brief anaphylactic symptoms which in 2 of the animals were so slight that the reaction must be called doubtful. The remaining guinea-pig, F 31, that was given 0.1 ml normal serum intravenously, showed almost the same anaphylactic symptoms as F 26 and F 27, which were challenged with schizophrenic serum. Therefore, it is possible that the slight anaphylactic reactions in all the guinea-pigs are due to deficient desensitization.

TABLE 2
Experiment 1

	Sensitizing	Desensitizing					Challenge	
Date	Sept 4	Sept 26	Sept 27	Sept 27	Sept 28	Sept 29	Sept 30	
Guinea pig No	S P 1-7 0.5 ml s c	N H S 0.5 ml s c	N H S 0.2 ml i v	N H S 0.5 ml s c	N H S 0.1 ml i v	N H S 0.1 ml i v	S P 1-7 0.1 ml i v	N H S 0.1 ml i v
F 26	0	0	–	0	3	0	3	–
F 27	0	0	–	0	2	0	3	–
F 28	0	0	–	0	4	1	0	
F 29	0	0	–	0	4	0	1	
F 30	0	0	5					
F 32	0	0	–	0	3	2	1	–
F 31	0	0	–	0	4	3	–	3

S P 1–7 = pooled sera from schizophrenic patients Nos 1 2 3 4 5 6 and 7
N H S = normal human serum
s c = subcutaneously
i v = intravenously
0 = no reaction
1 = doubtful reaction
2 = faint reaction
3 = significant reaction
4 = strong reaction
5 = died in anaphylactic shock
– = no injection

Experiment 2 (cf Table 3)

In this experiment we sensitized the guinea-pigs, like *Malis et al.*, with one subcutaneous injection of 0.1 ml serum from schizophrenic patients. In experiment 1 we used pooled sera from 7 schizophrenic patients for the sensitization as well as in the shock test. In the present experiment we used the pooled sera from Cases 6 and 7, which gave some reaction in the intestinal contraction experiments.

In this experiment, too, the desensitization gave rise to greater difficulties than reported by *Malis et al.* (cf guinea pig F 34 and the reactions following the first and second intravenous desensitization).

It is evident from the experiment that 7 out of 11 guinea-pigs which received 0.15 ml patient serum intravenously showed no anaphylactic symptoms or doubtful reactions. Four of the guinea pigs (*F 37, 41, 45,*

and 46) showed definite, though slight, anaphylactic reactions, and these 4 guinea pigs exhibited the same reactions when 24 hours later they received an intravenous injection of 0.15 ml normal human serum. It is very likely, therefore, that these 4 guinea pigs had not been completely desensitized. Two guinea-pigs (F 33 and 44), that received an *intravenous injection of* 0.15 ml normal human serum, instead of schizophrenic serum, showed no symptoms.

TABLE 3
Experiment 2

	Sensitizing	Desensitizing					Challenge		Control
Date	Dec 18	Jan 8	Jan 9	Jan 9	Jan 10	Jan 11	Jan 12	Jan 12	Jan 13
Guinea pig No	S P 6-7 0.1 ml s c	N H S 0.5 ml s c	N H S 0.15 ml i v	N H S 0.5 ml s c	N H S 0.15 ml i v	N H S 0.15 ml i v	S P 6-7 0.15 ml i v	N H S 0.15 ml i v	N H S 0.15 ml i v
F 34	0	0	5						
F 35	0	0	–	0	2	1	0	–	
F 36	0	0	–	0	2	1	0		–
F 37	0	0	–	0	3	2	3		3
F 38	0	0		0	3	0	0		–
F 39	0	0	–	0	1	0	0	–	
F 40	0	0	–	0	4	3	1	–	–
F 41	0	0		0	2	1	3	–	3
F 42	0	0		0	2	0	0	–	–
F 43	0	0		0	2	0	0	–	–
F 45	0	0	–	0	4	3	3	–	3
F 46	0	0		0	4	1	3	–	3
F 33	0	0		0	4	4	–	0	–
F 44	0	0		0	2	0	–	0	–

S P 6-7 = pooled sera from schizophrenic patients Nos 6 and 7
Other abbreviations and signs cf Table 2

TABLE 4
Experiment 3

	Sensitizing	Desensitizing				Challenge		
Date	May 28	June 18	June 19	June 20	June 21	June 22	June 22	June 23
Guinea-pig No	S 31 0.1 ml s c	N H S 0.5 ml s c	N H S 0.5 ml s c	N H S 0.15 ml i v	N H S 0.15 ml i v	S 31 0.15 ml i v	N H S 0.15 ml i v	S 31 0.15 ml i v
F 47	0	0	0	3	3	–	1	2
F 48	0	0	0	3	0	0	–	–
F 49	0	0	0	5				
F 50	0	0	0	3	0	0	–	–

S 31 = serum from schizophrenic patient No 31
Other abbreviations and signs cf Table 2

Experiment 3

Since the sera used in these two experiments were derived from patients with disease of long standing, we performed a minor experiment using serum from a recently diagnosed case of acute schizophrenia (serum No 31) From Table 4 it will be seen that the control guinea-pig (F 47) showed a doubtful reaction, and that the 2 *guinea-pigs that received* 0 15 ml schizophrenic serum showed no reaction

TABLE 5
Experiment 4

Date	Sensitizing	Desensitizing	Challenge	Control
	Oct 25	Nov 19	Nov 19 2 hours later	Nov 19 2 hours later
Guinea pig No	0 1 ml S P 32 34 s c	0 05 ml N H S i v	0 15 ml S P 32 34 i v	0 15 ml N H S i v
F 51	0	4		3
F 52	0	4		2
F 53	0	4	2	
F 54	0	2	1	
F 55	0	3		3
F 56	0	5		
F 57	0	3	2	
F 58	0	2	2	
F 59	0	2	2	
F 60	0	1		1
F 61	0	2		2
F 62	0	1	0	

S P 32–34 = pooled sera from schizophrenic patients Nos 32 33 and 34
Other abbreviations and signs cf Table 2

Experiment 4 (cf Table 5)

Lastly, we tried the technique described in *Malis et al's* third and fourth experimental series We sensitized 12 guinea pigs by subcutaneous injection of 0 15 ml serum from schizophrenic patients, and 25 days later they were desensitized by a single intravenous injection of 0 05 ml normal human serum Two hours later we tested 6 of the guinea-pigs with 0 15 ml of schizophrenic serum injected intravenously, while 5 were tested with 0 15 ml of normal human serum intravenously

From Table 5 it will be seen that all the guinea pigs that received serum from schizophrenic patients as well as those tested with normal human serum, exhibited uniform slight anaphylactic reactions

As a conclusion of experimental series A we can state that we were unable to reproduce *Malis et al's* and *Haddad & Rabe s* results The weak reactions observed in some of the guinea-pigs following injection of serum from schizophrenic patients are presumably due to the dif

ficulty of completely desensitizing guinea pigs that have been sensitized by human serum

This is also apparent from the following experimental series

Experimental Series B

In order to demonstrate the antigen in the blood of schizophrenic patients found by *Malis et al* we used the Schultz Dale method

Technique

The sensitized guinea pigs were killed by inhalation of chloroform whereupon as much blood as possible was tapped by cardiac puncture About 10 cm of the distal end of the ileum which gives the minimum number of spontaneous contractions was removed and stored after rinsing in oxygenated Ringer s solution at 37° C Suitable strips of this intestine were suspended by a thin cotton thread in Dale bath containing constantly oxygenated Ringer s solution at 37° C The movements of the gut were recorded kymographically The response of the strips of intestine to a standard dose of 0 15 μg histamine acid phosphate was observed before and after each series of test After having been thoroughly washed with Ringer s solution it is rendered refractory to normal human serum diluted with physiological saline to 1 5 or 10 per cent When the refractory state has been attained the intestine is tested with the same dose of serum from a schizophrenic patient

Experiment 1

In this experiment we used a stronger sensitization with schizophrenic serum than in the shock experiments (cf Table 6)

Some guinea pigs were sensitized by serum from schizophrenic patients treated with chlorpromazine (F 1 9) Others (F 11 12 and 13) were sensitized with serum from schizophrenic patients who had never been treated with chlorpromazine For control purposes we used guinea pigs (F 16 and 17) sensitized with serum from patients with mental diseases other than schizophrenia and treated with chlorpromazine

The sensitized guinea pigs were killed not earlier than one week after the injection of serum Strips of the ileum were suspended in the Schultz Dale apparatus and rendered refractory to normal human serum When this had been attained serum from schizophrenic patients was added

The result may be seen from Table 6 Out of the total of 8 guinea pigs (1 1 2 3 4 5 6 8 and 9) sensitized by serum from chlorpromazine treated schizophrenic patients and rendered refractory to normal serum 3 gave no contraction following exposure to schizophrenic serum Five showed in some cases an indication of intestinal

TABLE 6

Guinea pig No	Sensitization		Schultz Dale Test												
	Serum Lt No	0 1 ml subc Date	Date	Intestine No	Histamine No	Normal human serum					Challenge serum				
						Dose ml	Exposure No				Lt No	Dose ml	Exposure No		
							1	2	3	4			1	2	3
F 1	1 7	Sept 1 8 15 22 29	Oct 9	1	25	0 1	37	8	0		1-7	0 1	3	6	
				2	11	0 01	33	2			1 7	0 01	4		
				3	28	0 01	29	3			1-7	0 01	0		
				4	25	0 01	35	1			1-7	0 01	3	8	2
				5		0 0025	11	0 005 4	0 01 2		1 7	0 01	0	0	
				6		0 01	20				1-7	0 01	8	3	
				7		0 1	18	0			1-7	0 1	1	0	
F 2	1 7	Sept 1 8 15 22 29	Oct 16	1	10	0 1	42	6			1-7	0 1	2	2	
				2	8	0 01	19	3	0		1-7	0 01	0		
				3	19	0 1	31	13	1		1-7	0 1	4	1	
				4	22	0 1	35	8a	1		1	0 1	0		
				5	16	0 1	25	2	0		2	0 1	0		
				6	19	0 1	25	0			3	0 1	0		
				7	16	0 1	30	5	0		22	0 1	1		
F 3	1 7	Sept 1 8 15 22 29 Oct 6	Oct 17	1	0	0 01	27	2	0		1-7	0 01	0		
				2	17	0 1	33	3			1-7	0 1	0		
				3	20	0 1	36	1			1-7	0 1	3		
				4	27	0 1	35	3			22	0 1	7a	5a	
				5	20	0 1	34	1			23	0 1	3a		
				6	9						1 7	0 1	18	0	
F 4	1 7	Sept 1 8 15 22 29 Oct 6	Oct 17	1	8	0 1	44	10	6	4	1 7	0 1	10	9	6
				2	20	0 01	40	10	0		1 7	0 01	0		
				3	22	0 1	38	2			4	0 1	2		
				4	17	0 1	31	1			5	0 1	7	3	
				5	18	0 1	32	5	3		1 7	0 1	3		

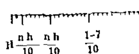
F 5	1 7	Sept	O t 24	1	6					1 7	0 1	36	0
		1 8 1		2	1	0 1	40	0		1 7	0 1	2	
		22 23		3	20	0 1	38			1 7	0 1	0	
		O t 6		4	17	0 1	32			1 7	0 1	0	
F 6	1 7	Sept	O t 24	1	10	0 1	41	J		1 7	0 1	10a	0
		1 8 15		2	18	0 1	44	0		1 7	0 1	9	
		22 21		3	15	0 1	30	2		7	0 1	18	
		O t 6		4	9	0 05	29	20	J	6	0 05	7	
				5	8	0 05	42	2		7	0 05	3	
F 8	1 7	Sept	N v 6	1	0	0 1	32	2		1 7	0 1	1	
		1 8 15		2	3	0 0	26	0		1 7	0 05	0	
		22 23											
		O t 6											
F 9	1 7	Sept	Dec 4	1	7	0 1	36	0		24	0 1	0	
		1 8 15		2	15	0 1	32	0		1 7	0 1	0	
		22 23											
		Oct 6											
F 11	8 12	Sept	Oct 18	1	7	0 1	25	0		8 12	0 1	6a	0
		1 8 15		2	11	0 1	21	0		1 7	0 1	0	
		22 23		3	16	0 1	30			8 12	0 1	0	
				4	14	0 1	32			8	0 1	15a	
				5	16	0 1	21	0		J	0 1	0	
				6	24	0 1	30			10	0 1	12	
				7		0 1	18	0		10	0 1	2	
F 12	8 12	Sept	O t 25	1	12	0 1	43	0		8 12	0 1	0	
		1 8 15		2	10	0 1	34	0		1 7	0 1	0	
		22 23		3	11	0 1	32	0		8 12	0 1	1	
		Oct 6		4	10					8 12	0 1	28	
F 13	8 12	Sept	Oct 27	1	1	0 1	35	0		8 12	0 1	2	
		1 8 15		2	17	0 1	40			8 12	0 1	3	
		22 23		3	17	0 1	31			1 7	0 1	0	
		Oct 6											

TABLE 6 (cont.)

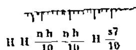
Guinea pig No	Sensitization		Schultz Dale Test												
	Serum Pt No	0 1 ml subc Date	Date	Intestine No	Histamine No	Normal human serum					Challenge serum				
						Dose ml	Exposure No				Pt No	Dose ml	Exposure No		
							1	2	3	4			1	2	3
F 16	16–19	Sept , 1 , 8 , 15 , 22 , 29 , Oct , 6	Oct , 25	1	6	0 1	42	18	9	5	16–19	0 1	4		
				2	14	0 05	33	10	0		16–19	0 05	3a		
				3	16						16–19	0 1	32	0	
				4	11	0 1	32	1			1–7	0 1	2		
F 17	16 19	Sept 1 , 8 15 , 22 29 ,	Oct , 27	1	23	0 1	54	9	10		16–19	0.1	5		
				2	15	0 05	30	5			16–19	0 05	0		
				3	25	0 1	40				1–7	0 05	5		
				4	13						16–19	0 1	38	0 05 1	
				5	11	0 1	27	3			16–19	0 1	1		
		1 ml subc													
F 21	1 7	Sept , 8 , 22	Oct 17	1	15	0 1	38	0			1–7	0.1	0		
				2	14	0 1	31	0			1–7	0 1	0		
				3	20	0 1	26	0			5	0 1	0		
				4		0 005	17	0 05 6	0 1 0		5	0 1	0		
				5		0 1	25				1–7	0 1	0		
I 24	1 7	Sept 8	Oct 17	1	1	0 1	20	0			1–7	0 1	0		
				2	21	0 1	31				1–7	0 1	0		
				3	19	0 1	26				5	0 1	1		

Serum Pt No 1 7 = pooled sera from schizophrenic patients Nos 1, 2, 3, 4, 5, 6, and 7
Serum Pt No 8 12 = pooled sera from schizophrenic patients Nos 8, 9, 10, 11, and 12
Serum Pt No 16 19 = pooled sera from patients Nos 16, 17, 18, and 19
a — late and atypical contraction
The figures in the columns 6, 8 9 10 11, 14, 15, and 16 indicate the size of the contraction in mm



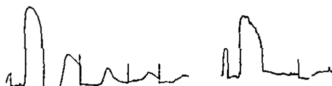


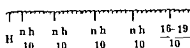
H $\frac{nh}{10}$ $\frac{nh}{10}$ $\frac{1\text{-}7}{10}$



H H $\frac{nh}{10}$ $\frac{nh}{10}$ H $\frac{s7}{10}$

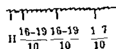
Fig 1





H $\frac{nh}{10}$ $\frac{nh}{10}$ $\frac{nh}{10}$ $\frac{nh}{10}$ $\frac{16\text{-}19}{10}$

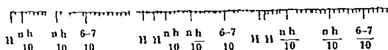
Fig 2 a



H $\frac{16\text{-}19}{10}$ $\frac{16\text{-}19}{10}$ $\frac{1\text{-}7}{10}$

Fig 2 b





H $\frac{nh}{10}$ $\frac{nh}{10}$ $\frac{6\text{-}7}{10}$ H H $\frac{nh}{10}$ $\frac{nh}{10}$ $\frac{6\text{-}7}{10}$ H H $\frac{nh}{10}$ $\frac{nh}{10}$ $\frac{6\text{-}7}{10}$

Fig 3

H = 1 1 ml of histamine phosphate sol ~ 1 µg histamine 1 ml.

$\frac{nh}{10}$ $\frac{1}{10}$ ml of normal human serum

$\frac{1}{10}$ $\frac{1}{10}$ ml of pooled sera from patients Nos 1 7

$\frac{6\text{-}}{10}$ $\frac{1}{10}$ ml of pooled sera from patients Nos 6–7

$\frac{16\text{-}19}{10}$ $\frac{1}{10}$ ml of pooled sera from patients Nos 16–19

$\frac{s}{10}$ $\frac{1}{10}$ ml of serum from patient No 7

TABLE 6 (cont)

Guinea pig No	Sensitization		Schultz Dale Test												
	Serum Pt No	0 1 ml subc Date	Date	Intestine No	Histamine No	Normal human serum					Challenge serum				
						Dose ml	Exposure No 1	2	3	4	Pt No	Dose ml	Exposure No 1	2	3
I 16	16 19	Sept 1 8 15 22 29 Oct 6	Oct 25	1	6	0 1	42	18	9	5	16 19	0 1	4		
				2	14	0 05	33	10	0		16 19	0 05	3a		
				3	16						16 19	0 1	32	0	
				4	11	0 1	32	1			1-7	0 1	2		
F 17	16 19	Sept 1 8 15 22 29	Oct 27	1	23	0 1	54	9	10		16 19	0 1	5		
				2	15	0 05	30	5			16 19	0 05	0		
				3	25	0 1	40				1 7	0 05	5		
				4	13						16 19	0 1	38	0 05 1	
				5	11	0 1	27	3			16 19	0 1	1		
		1 ml subc													
F 21	1 7	Sept 8 22	Oct 17	1	15	0 1	38	0			1 7	0 1	0		
				2	14	0 1	31	0			1 7	0 1	0		
				3	20	0 1	26	0			5	0 1	0		
				4		0 005	17	0 05 6	0 1 0		5	0 1	0		
				5		0 1	25				1 7	0 1	0		
I 24	1 7	Sept 8	Oct 17	1	1	0 1	20	0			1 7	0 1	0		
				2	21	0 1	31				1 7	0 1	0		
				3	19	0 1	26				5	0 1	1		

Serum Pt No 1 7 — pooled sera from schizophrenic patients Nos 1 2 3 4 5 6 and 7
Serum Pt No 8 12 — pooled sera from schizophrenic patients Nos 8 9 10 11 and 12

Date	Intestine No	Histamine	Schultz Dale Test: Normal human serum: Dose ml	Exposure No 1 2 3 4	Challenge serum: Pt No	Dose ml	Exposure No 1
Jan. 13	1	3	0 1	21 1	6–7	0 1	1
	2	6	0 1	11	6 7	0 1	0
Jan 13	1	5	0 1	15 1	6–7	0 1	1
	2	7	0 1	13 0	6–7	0 1	0
Jan 12	1	2	0 1	6 2	6–7	0 1	0
	2	9	0 1	15 0	6–7	0 1	0
	3	17	0 1	18 0	6 7	0 1	0
Nov 19	1	10	0 1	30 6	32 34	0 1	5
	2	28	0 1	22 0	32 34	0 1	0
	3	18	0 1	15 0	32 34	0 1	0
	4	17	0 1	14 0	32 34	0 1	1
Nov 19	1	35	0 1	56	32 34	0 1	5a
	2	36	0 1	56	32–34	0 1	0

After having been rendered refractory to normal serum, the intestine from these guinea pigs showed a trace of reaction to serum 16–19, which had been used for sensitization (cf Fig 2a) In addition, there was also a trace of contraction of the refractory intestinal strips when exposed to the pooled sera from schizophrenic patients 1 7 (cf Fig 2b)

The last 2 guinea pigs in Table 6 were sensitized once and twice respectively by injection of 1 ml serum from schizophrenic patients After the intestines had been desensitized to normal serum, no contractions were observed upon exposure to serum from schizophrenic patients

Thus Experiment 1 shows that it may be difficult to distinguish between weak contractions due to antigen antibody reaction and purely

Guinea pig No	Sensitization Serum Pt No	Desensitization				
F 38	Dec 18 SP 6 7 0 1 ml s c	Jan 8 NHS 0 5 s c 0	Jan 9 NHS 0 5 s c 0	Jan 10 NHS 0 15 i v 3	Jan 11 NHS 0 15 i v 0	J. S 0
F 42	Dec 18 SP 6 7 0 1 ml s c	Jan 8 NHS 0 5 s c 0	Jan 9 NHS 0 5 s c 0	Jan 10 NHS 0 15 i v 2	Jan 11 NHS 0 15 i v 0	Ja S 0
F 44	Dec 18 SP 6 7 0 1 ml s c	Jan 8 NHS 0 5 s c 0	Jan 9 NHS 0 5 s c 0	Jan 10 NHS 0 15 i v 2	Jan 11 NHS 0 15 i v 0	Jan N 0 1
F 52	Oct 25 SP 32 34 0 1 ml s c	Nov 19 NHS 0 05 i v 4	Nov 19 2 h l NHS 0 15 i v 2			
F 61	Oct 25 SP 32 34 0 1 ml s c	Nov 19 NHS 0 05 i v 2	Nov 19 2 h l NHS 0 15 i v 2			

2 h l = 2 hours later
The figures in columns 3 4 5 6 and 7 indicate the size of the shock reaction (see Tab
The figures in columns 10 12 13 and 16 indicate the size of the concentration in mm
Other abbreviations and signs cf Table 2

contraction, but only in one (F 6) did the intestine respond by some contraction but not typical All reactions were tested several times and as a rule the weak reactions could not be reproduced on other guts from the same experimental animal To illustrate this experiment the maximum intestinal contraction obtained is shown in Fig 1

Three guinea pigs (F 11 12 and 13) were sensitized with serum from schizophrenic patients who had not been treated with chlorpromazine After the intestinal strips had been rendered refractory to normal serum there was a trace of a contraction in one case

Two guinea pigs (F 16 and 17) were sensitized with serum from patients with mental diseases other than schizophrenia and who had been treated with chlorpromazine

			Schultz Dale Test							
			Normal human serum					Challenge serum		
te	Intestine No	Histamine	Dose ml	Exposure No 1	2	3	4	Pt No	Dose ml	Exposure No 1
., 13	1	5	0 1	39	17	8	2	6–7	0 1	3a
	2	19	0 01	40	9	0		6–7	0 01	0
., 26	1	16	0 1	21	2			32–34	0 1	2a
	2	13	0 1	12	0			32 34	0 1	0
26	1	15	0 1	17	0			32 34	0 1	0
	2	28	0 1	30	0			32–34	0 1	0
o. 27	1	12	0 05	21	3			32–34	0 05	2
	2	15	0 05	28	5			32–34	0 05	0

Thus, these experiments show very clearly that the *Schultz-Dale* method for demonstrating antigen is far more sensitive than the shock method In addition, it is more reliable, as it is possible to ascertain, on the same intestinal strip, that it has become refractory In the shock experiment the residual sensitivity, demonstrated in spite of marked desensitization, presumably explains the rather weak reactions seen in a few of the animals in experimental series A.

DISCUSSION

As is evident from our experiments, we did not obtain the same results as *Malis et al* or *Haddad & Rabe*

This can hardly be due to differences in technique The sensitization of the experimental animals was performed in the way described by *Malis et al* But in the matter of desensitization with normal human serum *Malis et al's* and our findings disagree We could not, like *Malis et al* desensitize the animals by one injection, but had to use several, being in this respect in conformity with *Haddad & Rabe*

TABLE

Guinea pig No	Sensitization Serum Lot No	Desensitization				
I 33	Dec 18 SP 6 7 0 1 ml s c	Jan 8 NHS 0 5 s c 0	Jan 9 NHS 0 5 s c 0	Jan 10 NHS 0 15 i v 4	Jan 11 NHS 0 15 i v 4	Jan 12 NHS 0 15 i v 0
I 51	Oct 25 SP 32 34 0 1 ml s c	Nov 13 NHS 0 05 i v 4	Nov 19 2 h l NHS 0 15 i v 3			
I 55	Oct 25 SP 32 34 0 1 ml s c	Nov 13 NHS 0 05 i v 3	Nov 19 2 h l NHS 0 15 i v 3			
I 60	Oct 25 SP 32 34 0 1 ml s c	Nov 13 NHS 0 05 i v 1	Nov 19 2 h l NHS 0 15 i v 1			

Abbreviations and signs cf Table 7

non specific reactions Therefore a reaction described as trace can hardly be attributed with any importance especially as the contraction could frequently be reproduced in the same intestinal strip using the same serum

Experiment 2

In this experiment guinea pigs sensitized with serum and desensitized with serum injected subcutaneously and intravenously which failed to give any anaphylactic reaction showed a fairly pronounced reaction of the gut exposed to normal human serum (Table 7 Fig 3)

Experiment 3

In this experiment we tested the guinea pigs used previously in experimental series A by the *Schultz Dale* technique

The result shows that there was not a single case of definite contractions after the intestine had been rendered refractory by normal human serum (Table 8)

Date	Intestine No	Histamine	Schultz Dale Test — Normal human serum: Dose ml	Exposure No 1	2	3	4	Challenge serum: Lt No	Dose ml	Exposure No 1
...r., 13	1	5	0 1	39	17	8	2	6–7	0 1	3a
	2	19	0 01	40	9	0		6–7	0 01	0
... 26	1	16	0 1	21	2			32–34	0 1	2a
	2	13	0 1	12	0			32 34	0 1	0
..., 26	1	15	0 1	17	0			32 34	0 1	0
	2	28	0 1	30	0			32 34	0 1	0
...v 27	1	12	0 05	21	3			32 34	0 05	2
	2	15	0 05	28	5			32 34	0 05	0

Thus, these experiments show very clearly that the *Schultz-Dale* method for demonstrating antigen is far more sensitive than the shock method In addition, it is more reliable, as it is possible to ascertain, on the same intestinal strip, that it has become refractory In the shock experiment the residual sensitivity, demonstrated in spite of marked desensitization presumably explains the rather weak reactions seen in a few of the animals in experimental series A

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This might be due to a stronger sensitization of our experimental guinea-pigs than of those used by *Malis et al.* and a consequently greater difficulty in desensitizing But this is not indicated by the fact that *Malis et al.* could induce even a very strong hypersensitivity to the specific antigen said to be contained in the blood of schizophrenic patients Since this specific antigen can hardly be present in large quantities in the serum, it is surprising that it can sensitize the experimental animals to such an extent that they die in anaphylactic shock when challenged This presupposes that the animals are easy to sensitize

That *Malis et al.* desensitized the experimental animals by one injection is evident from several control experiments in which, for instance, they sensitized and desensitized the animals with normal serum and obtained no reaction upon injection of serum from schizophrenic patients It is very unlikely that the difference between *Malis et al's* and our results could be due to a difference in the subjective assessment of the anaphylactic symptoms, because a number of his experimental animals died in shock It must be admitted, therefore, that we can find no technical cause for the divergences between the results obtained by *Malis et al*, *Haddad & Rabe*, and ourselves

Our own investigations also gave a negative result on employment of the more sensitive technique of *Schultz-Dale* for demonstrating the specific antigen.

It is interesting to note that with this technique we could demonstrate that complete desensitization of the experimental animals is hardly obtainable This was evident from the following experiments Sensitized guinea-pigs, submitted to protracted desensitization by several subcutaneous and intravenous injections, and who did not show anaphylactic reactions in the shock experiments, were killed 2 hours after the injection of the challenge dose, and their intestines were tested by the *Schultz-Dale* method In all these experiments we obtained a definite anaphylactic intestinal contraction following exposure to normal serum Moreover, immediately after the experiment we examined intestinal specimens from 2 guinea pigs that had been desensitized and tested on the same day at an interval of 2 hours The intestinal specimens from these animals still gave a strong reaction to normal human serum The few and doubtful reactions observed in the shock experiments and in the experiments performed with the *Schultz-Dale* method, therefore, are presumably due to a slight residual sensitivity despite the desensitization That the *Schultz-Dale* method is well suited for demonstrating a possible specific antigen in the serum is apparent from previous experiments in which we have used this technique for analysing antigens in bacterial extract Thus, we could differentiate 4 different antigens in a mixture in which some of the antigens were present in small quantities In order to ascertain whether the *Schultz-Dale* method is well suited for demonstrating small amounts of a specific antigen in patient sera, we have started some model experiments in order to find

out how small amounts of such an antigen, added to the serum, can be demonstrated

Another possibility which has occurred to us is a difference in the patient series used by *Malis et al* and by ourselves

In the study of schizophrenia it is always difficult to know whether the series are comparable partly because of considerable diagnostic

must be assumed that different biological factors may induce the same mental syndrome In other words, it cannot be taken for granted that the same aetiological and pathogenetic factor is demonstrable in all patients presenting a schizophrenic syndrome

Cases of chronic psychoses of a schizophrenic symptomatology of many years duration and with many years of hospitalization are generally diagnosed everywhere as schizophrenic, but unfortunately the authors have far too seldom stated how the schizophrenia has been diagnosed Regarding their patients, *Malis et al* merely state "All the patients tested were suffering from a chronic form of schizophrenia, at the time of the investigation an exacerbation of the disease was noted" *Haddad & Rabe* describe their patients merely as chronic schizophrenics

For the present investigation we selected a material of women suffering from chronic schizophrenia of many years' duration, who had spent many years in hospital having active symptoms and a symptomatology as uniform as possible and of a nature which everywhere would no doubt be classified as schizophrenic This affords the greatest possibility of a biologically uniform material If we assume (although it is not absolutely certain) that *Malis et al* s and *Haddad & Rabe* s series have been of the same nature as ours, it is peculiar that we could not confirm their findings

SUMMARY

In comprehensive studies *Malis et al* demonstrated an antigen occurring in the blood of schizophrenic patients Using the shock method employed by *Malis et al* as well as the more sensitive method of *Schultz Dale* we were unable to demonstrate this antigen The causes of this disagreement are discussed

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BRIEF REPORT

POLIO NEUTRALIZATION TESTS WITH $MgCl_2$ STABILIZED VIRUS

By *Inger Petersen & Herdis von Magnus*

In neutralization tests various dilutions of a serum are usually tested against a constant amount of virus Most laboratories aim at having 100 ID50 in the tests, *but it is often difficult to obtain this number of doses exactly* and if the actual *virus dosage deviates too much from the desired 100 ID50 the experiments must be* repeated

In studies with storage of attenuated type 1 Sabin polio vaccine it was confirmed *in our laboratory* that dilution of the vaccine to 'working dilution' (*i e* 200 000 TCID50 in 3 drops) in equal amounts of 2 M $MgCl_2$ and 0 5 per cent lactalbumin *hydrolyzate* (1) resulted in a product stable at +4° C *for months*

Subsequently experiments were *carried out in* order to see whether the accuracy in polio virus neutralization tests could be increased if the virus was stored +4° C diluted to about 10 times the working *dilution in 1 M* $MgCl_2$ In the routine neutralization tests the diluted virus would need one dilution step only before use

In preliminary experiments a *poor stability* of polio virus strains, *both virulent* and attenuated was observed when equal parts of saline and 2 M $MgCl_2$ were employed as diluent In contrast the stability was *found* to be excellent if the diluent consisted of equal parts of 0 5 per cent lactalbumin hydrolyzate *and* 2 M $MgCl_2$ The pH of this mixture was about *7 0* Virus has been kept in this way at +4° C for 6 months without any decline in titer (Unpublished data)

Such *stabilized* virus suspensions have been employed for a total of 80 *routine* neutralization tests in tissue culture with all 3 types of polio virus (30 experiments with type 1, 24 with type 2 and 26 with type 3) The results have been compared with corresponding numbers of neutralization tests where the stock viruses have been kept at -60° C and diluted to working dilution *(about 10 -4)* just before use

In both experimental series the stock viruses were titrated at least 5 times before being employed in the routine neutralization *tests* The titrations were performed *in 1 log* steps with 10 tubes per dilution in monkey kidney tissue culture cells from either cynomolgus or cercopithecus *monkeys In the* neutralization tests a variation in the virus dose between 40 and 250 TCID50 has been considered acceptable Both series of tests have—as mentioned—*been part of our* routine work and have not been carried out simultaneously They have however been performed by the same very experienced technical assistants

TABLE 1

TCID50 Obtained in Routine Neutralization Tests with Polio Virus

Stock virus	Virus type	No of tests in which the indicated TCID50 were obtained															No of tests	No of tests <40 or >250
		25	32	40	50	63	80	100	125	160	200	250	316	400	500	630		
Undil., kept at -60° C	1	2	1	2	1	2	6	1	3	6	2	1	1	1		1	30	6
	2			3	1	3	1	2	2	2	3	2	2	2		1	24	5
	3			1	1	2	3	3	6	3	2	2	1	1	1		26	3
10 -4, +4° C $MgCl_2$ and lactalb	1		1	3	2	5	3	8	5	2		1					30	1
	2		1	3	3	2	5	2	2	1	5						24	1
	3	1			4	3	2	3	4	5	1	3					26	1

Received 26 v 64 *from the Enterovirus Department Statens Seruminstitut Copenhagen, Denmark*

In Table 1 the results of the 2 sets of tests have been recorded It will be seen that in the series where undilutd stock virus kept at 60° C was used, 14 out of 80 experiments had to be repeated, whereas only 3 out of 80 tests had to be repeated when stabilized, diluted virus suspensions were employed

In our hands, the use of polio virus diluted in equal amounts of 2 M $MgCl_2$ and 0.5 per cent lactalbumin hydrolyzate presented the following advantages for use in routine neutralization tests in tissue culture Greater accuracy as regards dosage, simpler technic and less consumption of undiluted virus stock

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BRIEF REPORT

GEL PRECIPITATION WITH ANTIGENS PREPARED FROM HELA CELLS INFECTED WITH HERPES SIMPLEX VIRUS

By *Rauno Mäntyjarvi & Heikki Arstilommi*

Gel diffusion precipitation mostly as double diffusion on agar coated slides, has in the past few years been applied to studies of several viruses, including polio f ot and mouth chickenp
myxoviruses and adenovi
Gel precipitation with

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The present preliminary report describes gel precipitation between antigens prepared from HeLa cells infected with herpes simplex virus and human sera

Materials and methods HeLa cell monolayers in Roux bottles grown in Hanks' balanced salt solution with 40 per cent human serum and maintained with Eagle's Minimum Essential Medium with 5 per cent horse serum were infected with herpes simplex virus strain Kumpulainen In 1 to 2 days when more th
cells

cells ... an identical procedure from uninfected HeLa

They were sent to the

r pH 7 0 on 8 × 8 cm
aced 6 mm apart The

complement four units of antigen and overnight fixation at +4° C.

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Neutralization tests were performed with the same virus strain as the gel precipitation. 100 TCD$_{50}$ of virus was incubated with an equal volume of serum diluted 1:8 one hour at 37° C and then overnight at +4° C. Two HeLa cell tubes were inoculated with every serum-virus mixture.

Results. The 155 sera tested showed 0 to 3 precipitation lines with the herpes antigens used. *No precipitation lines were found with the control antigens.* As shown in Table 1, the presence and number of lines was correlated with the CF titer. Of the 35 specimens with the CF *titer* ≦ 1 : 4, 33 *were negative in precipitation and two* gave one precipitation line. On the other hand, each of the three specimens with *three precipitation lines* had a CF titer ≧ 1 : 128. The neutralization test was carried out on 110 of the sera. The sera having no neutralizing antibody were the same *giving a CF titer* ≦ 1 : 4. *One* serum with CF titer < 1 : 4 had neutralizing antibodies. The 19 sera that were negative *in neutralization were all negative in precipitation*.

Comments. The identity of the precipitating antigens is under further *investigation* but they are apparently herpes specific, since several antigen preparations showed no precipitation.

The reaction of human sera to herpes simplex precipitating antigens and to some other viruses seems to be similar. With *adenoviruses and human sera* 0 up to 2 or 3 precipitation lines can be obtained (*Tanaka* 1957; *Battistini & Ronchetti* 1961) and precipitation is highly correlated with the CF titer (*Derevici & Istrati* 1961). *Some* correlation between precipitation and CF was also obtained by *Taylor-Robinson & Rondle* (1959) with herpes zoster vesicle fluid and human sera. After poliomyelitis infection two antibodies giving precipitation have also been demonstrated by *Paccaud et al.* (1960). *The* same authors also found that after natural poliomyelitis infection the precipitating and neutralizing antibodies were simultaneously present in each case and the precipitating and CF antibodies *in 34 out 36 cases*.

TABLE 1

The Distribution of Herpes Complement Fixation Titers of 155 Human Sera with Respect to the Presence and Number of Precipitation Lines

Number of precipitation lines	CF titer						
	≦4	8	16	32	64	≧128	Total
0	33*	12	13	25	5	–	88
1	2	–	8	15	15	2	42
2	–	1	2	8	10	1	22
3				–	–	3	3
Total	35	12	23	48	30	6	155
% positive in precipitation	6	8	44	48	83	100	43

* Number of sera.

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BRIEF REPORT

ANTIMYCOBACTERIAL HUMORAL ANTIBODIES IN RATS WITH ADJUVANT ARTHRITIS

By P Toivanen J L Kalliomaki, H A Saarimaa & Auli Toivanen

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the subject

Ninety eight white adult male rats each weighing 240–300 g were divided into five groups One group served as non arthritic controls while in the four other [illegible] suspension of Mycobacterium phlei, as

by this agent (Hitchings & Elion 1963) Two groups were treated [illegible] caproic acid (100 mg and 50 mg/rat/day subcutaneously) This drug does to some

after the climax of the disease) and the rest a week later The sera were removed and stored at 20° C.

had been immunized with heat killed dried M phlei served as a positive control giving a titer of 1 40 with both antigens

Results In the group treated with 6 mercaptopurine the number of animals with arthritis was about the same as in the controls but the mean arthritis score was less than half that in the controls throughout the whole experiment Rats treated with ε aminocaproic acid developed arthritis which was on average more severe than in the 6 mercaptopurine group but a little milder than in the controls The effect of ε aminocaproic acid on arthritis is described in detail separately (Toivanen & Toivanen)

The number of rats with detectable antibodies in the various groups is given in the table Because of anticomplementary activity and the small volume of specimen some sera in each group [illegible] 5 to 1 160 and [illegible] oups nor in rats [illegible] e using both an [illegible] Tuberculin were

Received 9 ix 64 from the Department of Medical Microbiology (Head Professor F Mustakallio M.D) University of Turku Turku Finland

TABLE 1

Number of Rat Sera with Detectable Complement Fixing Antibodies/Number of Sera Examined in the Various Groups of Rats

Antigen	Non arthritic control rats	Arthritic rats treated daily with			
		3.0 mg 6-mercaptopurine	100 mg ε aminocaproic acid	50 mg ε aminocaproic acid	Physiologic saline
Old Tuberculin 1:100	1/13	7/20	2/13	6/16	3/19
M phlei 0.2 mg/ml	2/13	5/20	2/13	3/16	2/20

lacking while the titer of *M phlei* antibodies was up to 1:20 and *vice versa*. No correlation was noted between the antibody titer and the severity of the disease. The number of sera showing anticomplementary activity in a dilution 1:5 was greatest (6 out of 20) in the group of 100 mg of ε aminocaproic acid while in the other groups maximally 3 sera out of 19 were anticomplementary.

Antibodies against Old Tuberculin detected in the non arthritic control rats to the same extent as in the arthritic rats are a clear indication of the unspecific nature of these antibodies so far as the etiology of adjuvant arthritis is concerned. This indication is supported by the antibody frequency in rats treated with 6 mercaptopurine or ε aminocaproic acid. It has been demonstrated that rats with adjuvant arthritis develop cellular tuberculin hypersensitivity (*Waksman et al*; *Flax & Waksman*), and this can be inhibited by 6 mercaptopurine (*Kalliomaki et al*). Together with this fact the absence of humoral antibodies supports the opinion that the delayed type hypersensitivity is the acting mechanism in adjuvant arthritis. The present observations do not, however, permit of any conclusions as to the identity of the causative antigen in this disease.

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Å See Aa   Æ See Ae   Ä See Ae   Ö See Oe   Ø See Oe
